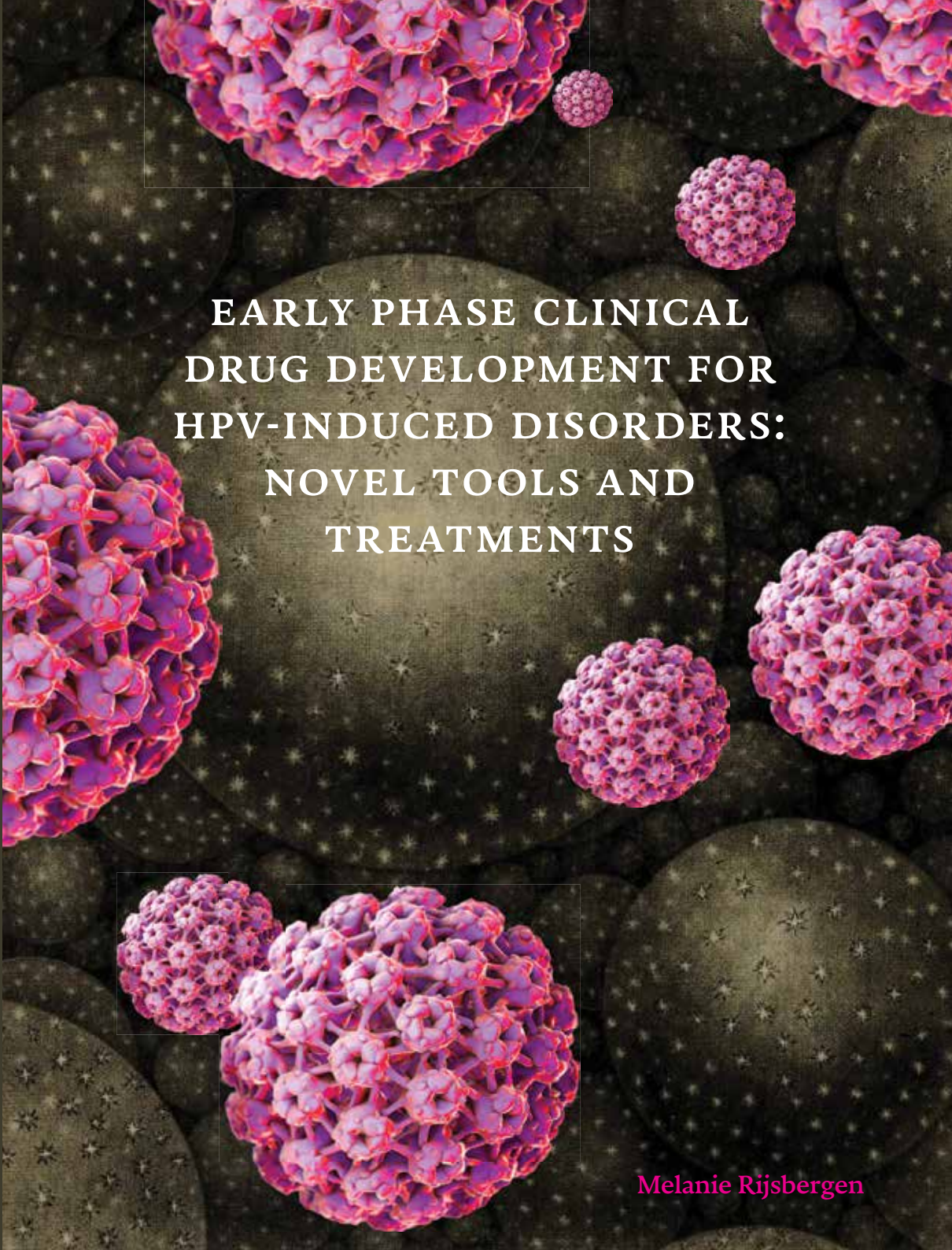


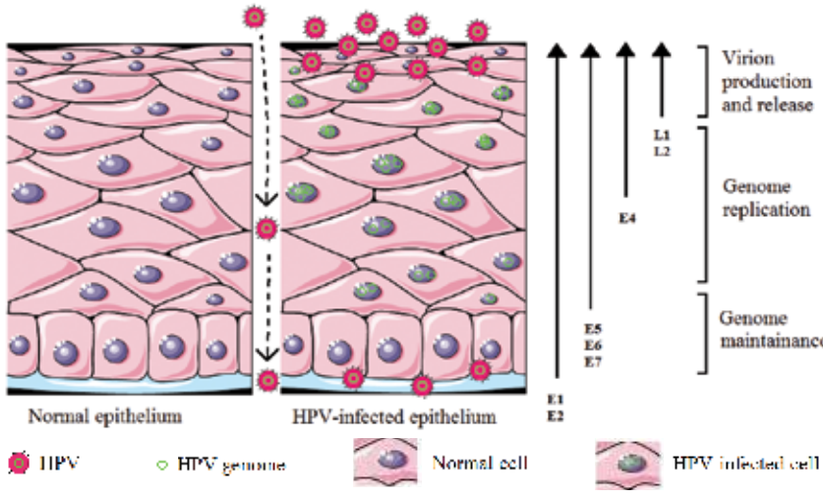
EARLY PHASE CLINICAL DRUG DEVELOPMENT FOR HPV-INDUCED DISORDERS: NOVEL TOOLS AND TREATMENTS

Melanie Rijsbergen

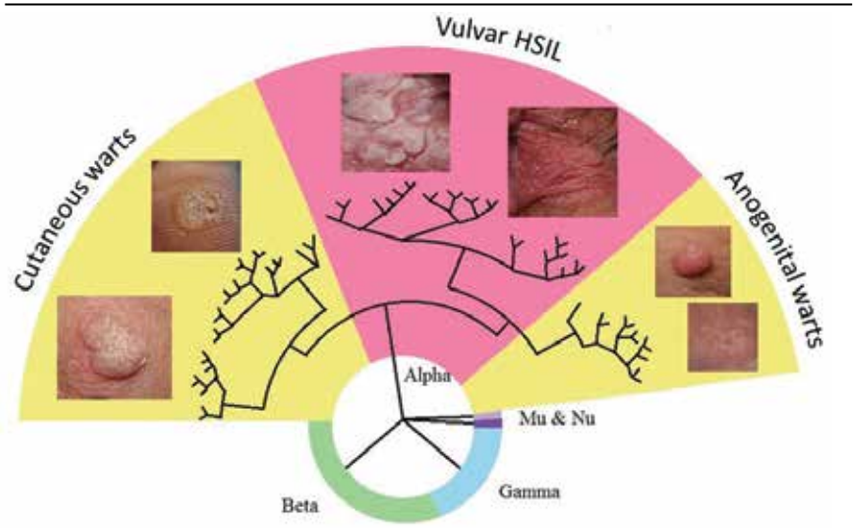
Chapter 1 – Figure 1. Genome organization of the Alpha papillomavirus HPV16. The genome is comprised of a long control region (LCR) and eight genes that are involved in the virus life cycle. This figure is adapted from de Sanjosé 2018 and Doorbar 2015.[9,10]

E1+E2:	Genome replication	E6+E7:	Oncogenes
E4:	Virus release and transmission	L1:	Major capsid protein
E5:	Evading immune response and apoptosis	L2:	Minor capsid protein
		LCR:	Long control region

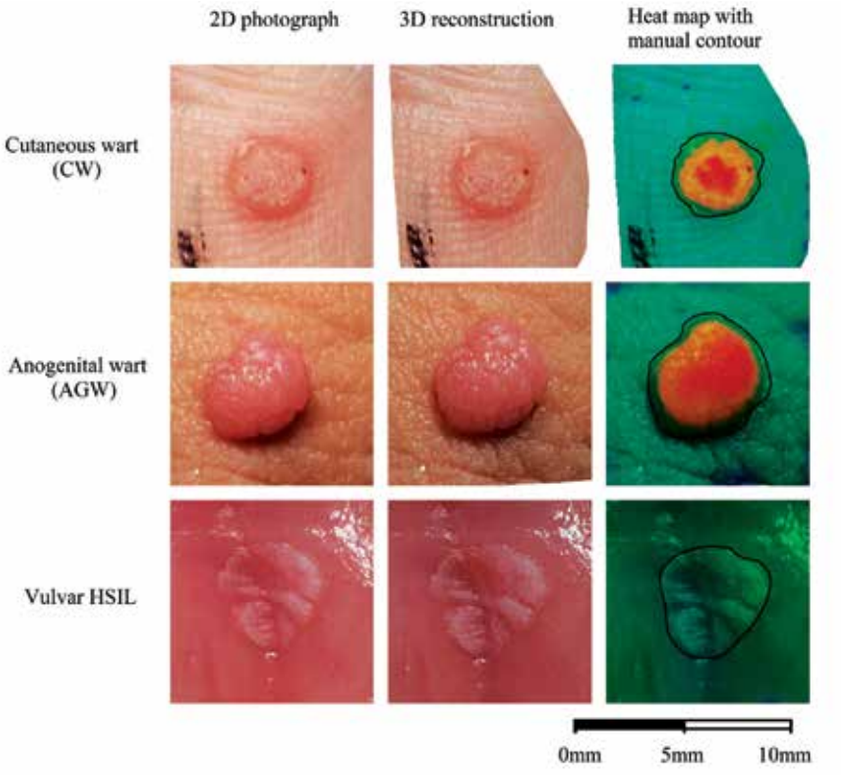
Chapter 1 – Figure 2. The life cycle of a HPV infection. A diagrammatic representation of the skin is shown after infection with HPV. Often a micro-trauma of the epithelium allows the virus to infect cells in the basal layer of the epithelium (dotted arrow lines). In the basal epithelial cells the virions are internalized and the viral genomes are transferred to the nucleus (genome maintenance). The genome of the virus is replicated in the nucleus and hereafter the virus particles are produced and released. The involvement of the early and late genes is shown with the arrows next to the figure. This figure is adapted from Doorbar 2005.[4]



Chapter 1 – Figure 3. Phylogenetic tree of human papilloma virus (HPV) demonstrating their evolutionary relationship. HPV types are divided in 5 different groups: Alpha (pink and yellow), Beta (green), Gamma (blue), Mu (purple) and Nu (lilac). The Alpha-papillomaviruses are subdivided as low-risk (yellow) and high-risk (pink) based on the benign or malignant potential of the virus, respectively. Cutaneous warts are caused by low-risk HPV types of the Alpha genus. Typical appearances of a common wart on the hand (left) and a plantar wart (right) are shown. Anogenital warts are also caused by low-risk HPV types of the Alpha genus, but these are phylogenetically different from the HPV types causing cutaneous warts as shown in the tree by the division of the branches. Anogenital warts on the penile shaft (upper) and under the foreskin (lower) are shown. High-risk HPV types of the Alpha genus (pink) cause vulvar high-grade squamous intraepithelial lesions (HSIL) with a high degree of variation in appearance, such as elevated hyperkeratotic white lesions (left) or red lesions (right).



Chapter 3 – Figure 1. 3D reconstruction of the twelve inch ruler (A) and wart-like object (B). Three-D reconstruction of the twelve inch ruler by the image reconstruction software (A), and the wart-like object in a 3D reconstruction with a heat-map showing the height of the object which is used for the 3D analysis (B).



SEE INSIDE FOR COLOR ILLUSTRATIONS OF CHAPTER 1 AND 3

EARLY PHASE CLINICAL DRUG DEVELOPMENT FOR HPV-INDUCED DISORDERS:
NOVEL TOOLS AND TREATMENTS

EARLY PHASE CLINICAL DRUG DEVELOPMENT FOR HPV-INDUCED DISORDERS: NOVEL TOOLS AND TREATMENTS

PROEFSCHRIFT

Ter verkrijging van de graad van Doctor
aan de Universiteit Leiden, op gezag van
Rector Magnificus prof. mr. C.J.J.M. Stolker,
volgens besluit van het College voor Promoties
te verdedigen op woensdag 19 februari 2020
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geboren te Leiderdorp in 1987

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Chapter 1

INTRODUCTION

Human papillomavirus (HPV) infections cause a variety of epithelial lesions ranging from benign common warts to malignant anogenital diseases. The overall disease burden of HPV infections varies correspondingly from minimal cosmetic discomfort to highly debilitating morbidity and even mortality. For the latter category the incidence of HPV-induced diseases still increases, even despite preventive vaccination strategies. As an example, the life-time risk of acquiring a genital HPV-infection is around 80% and almost 10% of these develop into persistent infections related to (pre)malignant diseases.[1,2] Current treatments of HPV-induced lesions consist of medical and surgical therapies that focus on lesion removal instead of eradication of the virus. These treatments are often associated with significant side effects and high recurrence rates. Therefore, there is a strong medical need for new HPV eliminating drugs with an acceptable side effect profile. The scope of this thesis is to elucidate novel pharmacological interventions for HPV-induced diseases by using a question-based developmental approach that includes investigation of the pharmacological effects in an early phase of drug development.[3] The emphasis of the thesis is on the development of new methodological tools to monitor the course of HPV-related diseases in clinical trials, as well as the exploration of successful biomarkers of viral load in HPV infections. This introduction summarizes the biology of HPV and its different types, the pathophysiology of HPV-induced diseases and addresses the relevance of question-based drug development and the development of new methodological tools and biomarkers.

HUMAN PAPILLOMAVIRUS BIOLOGY

Papillomaviruses are small, non-enveloped, circular, double-stranded DNA viruses that belong to the Papillomaviridae family and infect cutaneous and mucosal epithelial cells.[4] The genome of the HPV is distributed into early genes E1-E2, E4-E7 and late genes L1 and L2 (Figure 1).[5,6] The early genes E1, E2, E4 and E5 induce the maintenance, transformation and replication of the viral infection.[6] A persistent infection with a high-risk HPV type can induce the integration of the viral DNA into the human genome, causing overexpression of the E6 and E7 oncoproteins and lead to (pre)malignant progression of the lesion. The late genes encode for viral capsid proteins which are only expressed in highly differentiated epithelial cells and lead to a high number of HPV copies.

Figure 1. Genome organization of the Alpha papillomavirus HPV16. The genome is comprised of a long control region (LCR) and eight genes that are involved in the virus life cycle. This figure is adapted from de Sanjosé 2018 and Doorbar 2015.[9,10] *(see inside front-cover for image in color)*

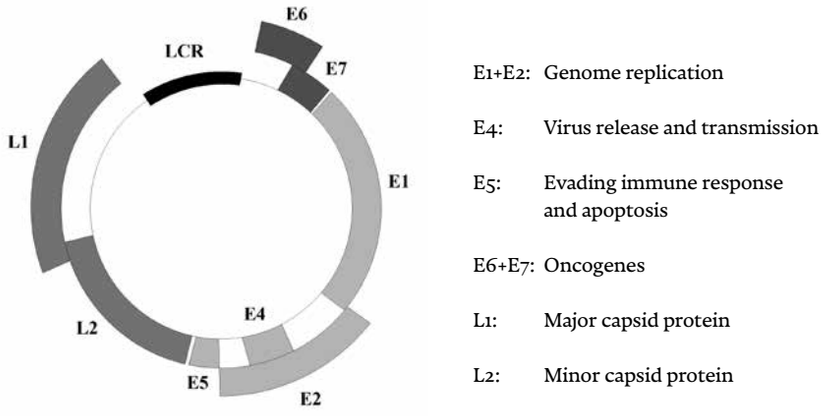
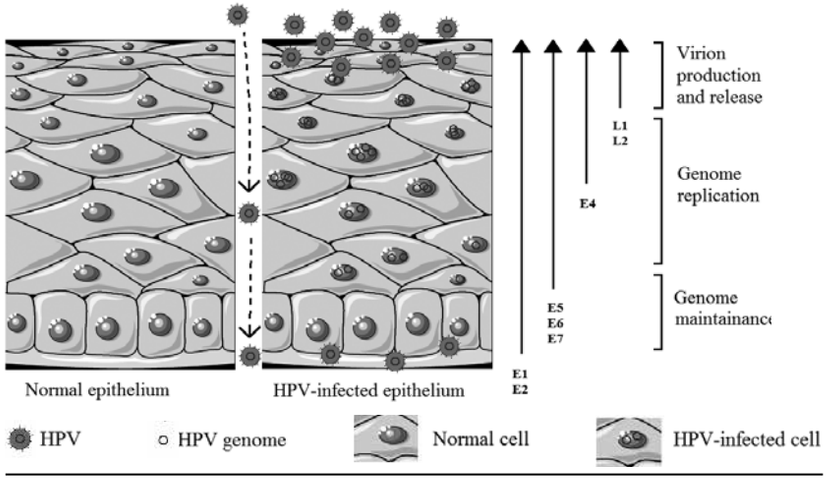


Figure 2. The life cycle of a HPV infection. A diagrammatic representation of the skin is shown after infection with HPV. Often a micro-trauma of the epithelium allows the virus to infect cells in the basal layer of the epithelium (dotted arrow lines). In the basal epithelial cells the virions are internalized and the viral genomes are transferred to the nucleus (genome maintenance). The genome of the virus is replicated in the nucleus and hereafter the virus particles are produced and released. The involvement of the early and late genes is shown with the arrows next to the figure. This figure is adapted from Doorbar 2005.[4] *(see inside front-cover for image in color)*



Infection with HPV requires access of the virus to cells in the basal layer, by micro trauma of the epithelium (Figure 2). The capsid of the virion is cleaved to facilitate the virus internalization.[6,7] Once the virions are internalized they undergo endosomal transport, further uncoating, cellular sorting and subsequent transfer of the viral genome to the nucleus. Infection of the lower epithelial layer is followed by an initial phase of genome amplification in low copy numbers and maintenance of the viral genome.[6] The genome amplification is further facilitated by the upper epithelial layers which results in a high genome copy number. It is hypothesized that the infection of an epithelial stem cell is necessary in the lesion formation process.[6,8]

To date, more than 207 different human papillomavirus (HPV) genotypes have been identified based on the DNA sequence and are divided into five phylogenetic groups: Alpha, Beta, Gamma, Mu and Nu.[5,11] Figure 3 shows the five phylogenetic groups and the subdivision of Alpha papillomaviruses into low-risk (yellow, e.g., cutaneous and anogenital warts) and high-risk (pink, e.g., vulvar high-grade squamous intraepithelial lesions (HSIL)) diseases.

Next to different life-cycle characteristics and disease associations, the HPV types in the different phylogenetic groups can be categorized as either cutaneous or mucosal genotypes based on their tissue preference (Table 1).

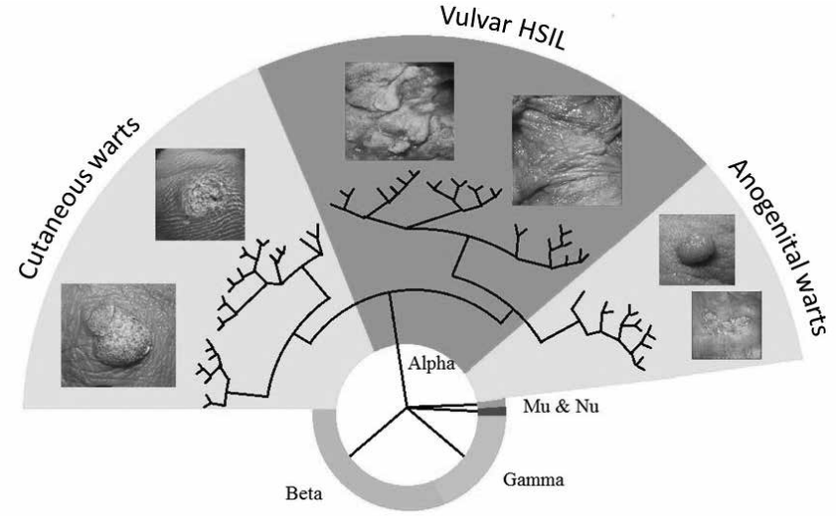
The majority of HPV infections manifest themselves as an asymptomatic infection of the cutaneous or mucosal epithelium, although self-limiting benign growth is also frequently observed. Most HPV types belong to the Alpha papillomaviruses group and these HPV types cause mucosal and cutaneous lesions. This group includes the HPV types that primary cause cutaneous warts and also the genitally transmitted HPV types. The latter mentioned viruses are the most common sexually transmitted pathogens worldwide and some of these are associated with the development of cancer.[4] The Beta papillomaviruses infect the cutaneous epithelia and mostly cause inapparent or latent infections but can also cause non-melanoma skin (pre)malignancies.[5] The Gamma, Mu and Nu papillomavirus mostly causes benign cutaneous lesions.[5]The subdivision of HPV types into low-risk and high-risk depends on their carcinogenicity, i.e. the benign or malignant potential of the virus.[14] Infection by low-risk HPV types is asymptomatic or can cause benign lesions. However, persistent infection with high-risk types can cause (pre)malignant anogenital lesions.[5] The lifetime risk of acquiring a genital HPV infection is about 80% and approximately 40% of female adolescents become infected with a high-risk HPV type at least once.[1,2,15] The prevalence of a high-risk infection decreases with age, being 15% in all women and 9% of all women above 30 years.[2] The majority of these infections are transient and will be cleared by the immune system without causing any lesions.

This thesis describes studies performed in patients with cutaneous warts, anogenital warts and vulvar HSIL, being Alpha papillomaviruses-induced diseases illustrative for typical low-risk (cutaneous warts and anogenital warts) and high-risk (vulvar HSIL) HPV-induced diseases falling within the scope of this thesis.

Table 1. The different human papilloma virus (HPV) groups based on their evolutionary relationship. The HPV types are divided in 5 different groups; Alpha (pink and yellow), Beta (green), Gamma (blue), Mu (purple) and Nu (lilac). The Alpha-papillomaviruses are subdivided as low-risk (yellow) and high-risk (pink) based on the benign or malignant potential of the virus, respectively. Examples of different HPV types, tissue preference and associated diseases are given.[12,13]

Genus + Species	Types	Tissue preference	Diseases
Alpha 1, 13	HPV32, HPV54	Mucosal	Low-risk mucosal lesions
Alpha 8	HPV7	Mucosal	Butcher's wart
Alpha 10	HPV6, HPV11	Mucosal/ cutaneous	Anogenital warts, oral/laryngeal papillomas
Alpha 9	HPV16, HPV31, HPV33	Mucosal	Vulvar and cervical HSIL/carcinoma
Alpha 7	HPV18, HPV45	Mucosal	Cervical HSIL/ carcinoma
Alpha 5, 6, 11	HPV51, HPV56, HPV34	Mucosal	Cervical HSIL/ carcinoma
Alpha 2, 3, 14, 15	HPV3, HPV10	Mucosal	Low-risk mucosal lesions, flat warts
Alpha 4	HPV2, HPV27, HPV57	Cutaneous	Cutaneous warts
Beta 1-5	HPV5, HPV8	Cutaneous	Skin cancer, epidermodysplasia verruciformis
Gamma 1-5	HPV4, HPV60	Cutaneous	Cutaneous warts, epidermoid cyst
Mu 1-2	HPV1, HPV63	Cutaneous	Cutaneous warts
Nu 1	HPV41	Cutaneous	Cutaneous lesions

Figure 3. Phylogenetic tree of human papilloma virus (HPV) demonstrating their evolutionary relationship. HPV types are divided in 5 different groups: Alpha (pink and yellow), Beta (green), Gamma (blue), Mu (purple) and Nu (lilac). The Alpha-papillomaviruses are subdivided as low-risk (yellow) and high-risk (pink) based on the benign or malignant potential of the virus, respectively. Cutaneous warts are caused by low-risk HPV types of the Alpha genus. Typical appearances of a common wart on the hand (left) and a plantar wart (right) are shown. Anogenital warts are also caused by low-risk HPV types of the Alpha genus, but these are phylogenetically different from the HPV types causing cutaneous warts as shown in the tree by the division of the branches. Anogenital warts on the penile shaft (upper) and under the foreskin (lower) are shown. High-risk HPV types of the Alpha genus (pink) cause vulvar high-grade squamous intraepithelial lesions (HSIL) with a high degree of variation in appearance, such as elevated hyperkeratotic white lesions (left) or red lesions (right). *(see inside front-cover for image in color)*



CUTANEOUS WARTS

Cutaneous warts are a common benign skin condition with an estimated prevalence of 3-13% in the general population in the Western world.[16] Most people are affected by cutaneous warts at some time point in their life.[16–19] The vast majority (>80%) of cutaneous warts in the general population is caused by HPV1, 2, 27 and 57.[20–25] Two-third of all warts show spontaneous regression within two years after diagnosis. Although cutaneous warts are benign and usually resolve spontaneously, they cause physical and psychosocial discomfort.[26,27] Cutaneous warts can be subdivided into plantar warts, located on foot soles, and common warts, located on all other skin locations (Figure 1). The diagnosis of cutaneous warts is based on clinical observations. Depending on the HPV-genotype and clinical location, the clinical appearance of common and plantar warts differs but most warts present as hyperkeratotic papules or plaques. Common warts are mostly elevated, scaly, rough and skin-colored papules or nodules, while plantar warts are mostly thick, hyperkeratotic, papules with capillary thrombosis.

The most frequently used treatments for cutaneous warts are cryotherapy, salicylic acid, ablation and surgical excision, aimed at destruction of the infected tissue. The efficacy, recurrence rates and side effects of these therapies are depicted in Table 2. The most commonly used therapy is cryotherapy which consists of the use of liquid nitrogen at a temperature of -196°C to create an area of necrosis below and around the wart.[28,29] Salicylic acid causes chemical ablation of the wart. Ablation with a laser or electrocautery has comparable efficacy rates as surgical excision, however is not often used because of the invasive nature and side effects.[28]

Table 2. Efficacy rates and side effects of the most common treatments for cutaneous warts.

Treatment	Efficacy rate	Side effects
Cryotherapy	29-51%[29–31]	Pain, blistering, scarring, irritation, pigmentation, crust formation[30]
Salicylic acid	16-35%[29–31]	Irritation, pain, blistering, bleeding, pigmentation[30]
Ablation	75-100%[29,31]	Pain, scarring, crust formation, irritation[29]

ANOGENITAL WARTS

Anogenital warts (AGW), caused by low-risk HPV types such as HPV6 and 11, are highly contagious and are the most common sexually transmitted viral disease worldwide with an incidence of 160-289 per 100.000.[1,32–34] Genital warts generally cause minor symptoms such as pruritus and irritation, but most patients report a high psychological burden from the disfiguring nature of the warts and concerns about infecting sexual partners.[35,36] Two prophylactic vaccines are available against HPV, namely 2-valent papillomavirus vaccine (Cervarix®) and 4-valent papillomavirus vaccine (Gardasil®). Cervarix® includes the high-risk HPV types 16 and 18 and Gardasil® includes next to HPV 16 and 18 also the low-risk HPV types 6 and 11. A 9-valent papillomavirus vaccine (Gardasil-9®) recently became available and contains 5 other high-risk

HPV types (31, 33, 45, 52, and 58) next to HPV 16 and 18. Since 2008, the Dutch national immunisation programme included the Cervarix® vaccine for girls aged 12-13. The success rate of immunisation is rather low as the coverage is only 45%.[37] In Australia the Gardasil® vaccine is included in the national immunisation programme and this has resulted in a dramatic reduction of the incidence of genital warts with almost 90%.[38] The diagnosis of AGW is a clinical diagnosis and no additional research, i.e. biopsy, is necessary. AGW can appear as solitary lesion, in clusters or as plaques and can be flat, dome-shaped, keratotic, pedunculated or cauliflower-shaped (Figure 3).

Current therapeutic options for AGW can be divided into topical therapeutical agents (podophyllotoxin, imiquimod) and surgical removal (cryotherapy, excision, electro surgery, laser ablation). Podophyllotoxin is a substance obtained from the rootstock of the may apple plant and causes cell death and destruction of the wart. Imiquimod is an immunomodulator which causes activation of different cytokines, particularly interferon-alpha. The efficacy and recurrence rates and side effects of these therapies are depicted in Table 3.

Table 3. Efficacy and recurrence rates and side effects of the most common treatments for anogenital warts.

Treatment	First/ second line	Efficacy rate	Recurrence rate	Side effects
Podophyllotoxin	First	45-48%[39–42]	4-38%[41,43]	Local inflammation or irritation, erosion, burning, pain, itching[44–46]
Imiquimod 5%	First	27-54%[47–49]	13-19%[48,49]	Erythema, erosion, itching, burning sensation[46,49]
Cryotherapyw	First	27-88%[50–53]	21-40%[51,52]	Pain, ulceration, scarring, irritation, pigmentation[51,52]
Surgical excision	First/second	35-72%[54,55]	19-29%[54,55]	Pain, scarring, crust formation[46,55]
Electro surgery	Second	61-94%[51,56]	22%[51,56]	Pain, scarring, irritation[46,51]
Laser therapy	Second	23-52%[29,46]	60-77%[46]	Pain, scarring, crust formation[46]

VULVAR HIGH-GRADE SQUAMOUS INTRAEPITHELIAL LESION

Vulvar high-grade squamous intraepithelial lesion (HSIL), previously known as usual vulvar intraepithelial neoplasia (uVIN), is a chronic premalignant vulvar skin disorder caused by a persistent high-risk HPV infection. Vulvar HSIL is in 90% of the cases caused by HPV type 16.[57] The incidence of vulvar HSIL is 2-5 per 100.000 but the incidence is rising and young women in their 30s and 40s are most often affected.[58,59] The majority of patients suffer from long-lasting and severe symptoms like pruritus or pain and the disease is associated with a high psychosocial burden and sexual dysfunction.[60] Vulvar HSIL has a malignant potential of 3-5% when treated, but when left untreated incidence may increase up to 9%.[57,61,62] Only up to 1.5% of vulvar HSIL lesions are reported to regress spontaneously.[61,62]

Vulvar HSIL has a highly variable appearance on clinical examination and may therefore be difficult to diagnose (Figure 3). Diagnosis always has to be confirmed by histopathological analysis. The lesions can be unifocal or multifocal, can present as plaques or papules and may be white, brown or red. The lesional skin can be thickened (hyperkeratosis), split (fissures) or ulcerated. Vulvar HSIL is often a multicentric disease, therefore it is important to examine the cervix, vagina and anus as well.[63]

The aim of vulvar HSIL treatment is symptom relief, restore normal anatomy and prevention of malignant progression. Considering the low malignant potential of the disease, expectative management in combination with close follow-up can be considered if patients have no severe symptoms and there is no suspicion of invasive disease. Treatment options for vulvar HSIL are surgical excision, ablation (laser therapy) and imiquimod (Table 4).

Table 4. Efficacy and recurrence rates and side effects of the treatments for vulvar HSIL.

Treatment	Efficacy rate	Recurrence rate	Side effects
Excision	Not reported	51%[60,64]	Disfiguring, pain[64,73]
Laser therapy	75%[62,74–76]	51%[60,64]	Not reported
Imiquimod	51-58%[64,65,67,77]	11-16%[65,66]	erythema, oedema, pain, erosion/ulceration, fatigue, headache, muscle pain[64–67,69,77]
Cidofovir	46%[69]	6%[70]	Pain, ulcera, fatigue, headache, muscle pain[69]

Further, the treatment is usually associated with psychosocial and sexual burden for the patient. Ablative techniques such as laser therapy offer increased precision and better cosmetic results and are as efficacious as local excision.[64] Surgical interventions aim to remove (excision) or destroy (laser) visible lesions, but do not eliminate the virus, while vulvar HSIL is caused by a persistent HPV infection. Therefore, there are often positive tumour margins

or still presence of HPV infection in the surrounding tissue after surgical intervention, resulting in a high number of recurrence and residual lesions of up to 40%.[64–66] Medical treatments have the advantage that there is minimal disruption of the anatomy compared to surgical interventions. The most commonly used medical treatment is imiquimod which was developed and licensed for anogenital warts but has also shown to be effective in vulvar HSIL.[66–68] Imiquimod is an immunomodulator that destroys abnormal cells by enhancement of the immune response of the body. Other treatments are cidofovir, photodynamic therapy and therapeutic vaccination. Cidofovir is an antiviral therapy and can be used as a local treatment for vulvar HSIL. A recent randomized clinical trial in 180 subjects performed to compare treatment with cidofovir and imiquimod in vulvar HSIL patients showed comparable efficacy and less recurrences in the cidofovir group.[69,70] Photodynamic therapy is based on light-induced oxidation reactions which lead to tissue necrosis and has the advantage that it is well tolerated and that the anatomy of the vulva is preserved.[71] Photodynamic therapy has been evaluated in small samples showing varying results in terms of efficacy.[64] Therapeutic vaccination with a synthetic peptide targeting specific HPV16 has shown to induce clinical responses.[72] Further investigation to evaluate the effect in treating vulvar HSIL is needed. Prophylactic vaccination against HPV has shown to reduce the risk of HPV-related diseases including vulvar HSIL.[64] The option of treatment differs per patient and depends on several factors such as location of the lesions, uni- or multifocality, comorbidity, previous treatment and obviously patient and physician preference.

DEVELOPMENT OF NOVEL THERAPEUTICS WITH NEW METHODS AND BIOMARKERS

Using the information on registered clinical trials as available in the trial registration 'clintrials.gov', 501 clinical trials for HPV-induced diseases were performed in the last decade. Of these studies, 79 were phase 1 trials, 125 phase 2, 83 phase 3, 42 phase 4, and 204 not assigned to a clinical phase. In July 2019, 208 clinical trials in HPV-induced diseases are executed according to the integrity database of Clarivate Analytics.[78] Of these trials, 80 are randomized and 55 are double-blind and placebo-controlled. These high numbers indicate the medical need for the development of new compounds for HPV-induced diseases. A likely explanation is that current treatments are not sufficient given their low efficacy and high recurrence rates.

The clinical development success rate, i.e. the likelihood that an experimental compound investigated in a phase 1 study is approved, was 9,6%, based on data from clinical trials performed in all disciplines from 2006 to 2015.[79] The transition success rate from phase 1 to 2 was 63.2% and from phase 2 to 3 30.7%.[79] Following the traditional four clinical phases of drug development, most early phase studies on new compounds explore safety and tolerability rather than human pharmacology of the specific drug.[80] The low clinical development success rate of approximately 1 out of 10 suggests that the early prediction of the efficacy and safety of a new compound is of crucial importance. Early signaling of limited efficacy could halt the further development of a compound which can save time and resources. However, the pharmacological effects of a new drug are often investigated in later stages of clinical drug development. Question-based development is a new approach integrating the evaluation of the pharmacological effects in an early phase of drug development.[3] By using this approach, specific questions guide the investigation of pharmacodynamics of a compound in an early stage of development. Questions obviously should be tailored to the compound type and indication(s) based on relevancy, but can generally be divided in 5 questions:[3]

1. Does the drug get to the site of action?
2. Does the compound cause its intended pharmacological/ functional effect(s)?
3. Does the compound have beneficial effects on the disease or its pathophysiology?
4. What is the therapeutic window of the drug?
5. How do the sources of variability in drug (e.g. dose, pharmacokinetics, and pharmacodynamics) response in the target population affect the development of the product?

It is essential to utilize the most appropriate methodology to answer these questions. Biomarkers are biological measures of pharmacodynamic drug effects. According to the World Health Organization, a biomarkers is 'any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease'.[81] The use of biomarkers in an early phase of drug development is of crucial importance to optimize development time and resources. A biomarker refers to a broad subcategory of medical signs of clinically significant patient outcomes that can be accurately and reproducibly measured.[82]

OUTLINE OF THIS THESIS

This thesis describes studies that address different aspects of early clinical phase drug development in three different HPV-related diseases. This thesis is divided into two parts: **section 1** describes the development and use of novel tools in clinical drug development and **section 2** focuses on early phase clinical studies examining safety, tolerability, pharmacodynamic and efficacy parameters of new topical compounds with high pre-clinical potential for the treatment of HPV-induced diseases.

SECTION 1 TOOLS AND BIOMARKERS IN EARLY PHASE CLINICAL TRIALS FOR HPV-INDUCED DISEASES

The clinical trials in this thesis employed new tools and biomarkers for three types of HPV-induced diseases (CW, AGW and vulvar HSIL) in an early phase of drug development with use of the question-based drug development approach. **Section 1** entails a thorough characterization of the implemented tools, including valid information about treatment adherence, (adverse) events and symptoms. **Chapter 2** describes the development, implementation and evaluation of an electronic diary to measure treatment adherence and patient-reported outcomes. **Chapter 3** entails the technical and clinical validation of a stereophotogrammetric 3D camera system.

SECTION 2 NOVEL TOPICAL TREATMENTS FOR HPV-INDUCED DISEASES

Section 2 describes the evaluation of two new topical compounds for HPV-induced diseases: i) omiganan, a small peptide known for its antimicrobial functions which has shown in vitro antiviral and immunomodulatory activity, and ii) ionic contra-viral therapy (ICVT) comprised of digoxin and furosemide which has shown in vitro antiviral activity. **Chapter 4** describes the results of two clinical trials using omiganan in patients with AGW and vulvar HSIL. The purpose of these trials was to assess the safety of omiganan and to explore pharmacodynamics and efficacy of omiganan in these diseases. In **Chapter 5** safety, pharmacodynamics and efficacy of topical ICVT are investigated in patients with cutaneous warts. **Chapter 6** presents the safety and efficacy of topical ICVT in patients with AGW in a randomized, vehicle-controlled trial.

Chapter 7 summarizes and discusses the overarching findings of these studies as well as future perspectives on drug development in HPV-induced diseases. This chapter includes a critical evaluation of the process of drug development and of novel tools and biomarkers in early phase clinical trials in HPV-induced diseases.

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SECTION I

TOOLS AND BIOMARKERS IN EARLY PHASE CLINICAL TRIALS FOR HPV-INDUCED DISEASES

Chapter 2

MOBILE E-DIARY APPLICATION FACILITATES THE MONITORING OF PATIENT-REPORTED OUTCOMES AND A HIGH TREATMENT ADHERENCE FOR CLINICAL TRIALS IN DERMATOLOGY

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Abstract

BACKGROUND Assessment of treatment effects in clinical trials requires valid information on treatment adherence, adverse events and symptoms. Paper-based diaries are often inconvenient and have limited reliability, particularly for outpatient trials.

OBJECTIVES To investigate the utility of an electronic diary (e-diary) application for patients with skin diseases in outpatient clinical trials.

METHODS An e-diary application was developed and technically validated. Treatment adherence as defined as topical administration by the patient and patient-reported outcomes, i.e. pain and itch, were evaluated by the e-diary in six clinical trials on newly tested topical drugs. Additionally, the proportion of patients capturing the applied topical drug by camera and filling in the pain and itch scores as defined as e-diary adherence, patients' perception of usefulness and acceptability of the e-diary were evaluated.

RESULTS Treatment adherence rates of the included 256 patients were high (median 98%, range 97-99%). E-diary adherence was also high with a median of 93% (range 87-97%) for capturing the applied drug by camera, 89% (range 87-96%) and 94% (range 87-96%) for entering respectively the itch and pain score. Daily symptom scores provided good insights in the disease burden and patients rated the e-diary as good to excellent with respect to user acceptability.

CONCLUSIONS The results suggest that the e-diary is an excellent way to ensure proper treatment administration, indicated by both the high user acceptability scores and high treatment adherence. Moreover, the e-diary may also be valuable for frequent and reliable monitoring of patient-reported outcomes in daily clinical practice.

Introduction

Treatment adherence is the degree to which patients take their medications as prescribed or as instructed by their treating physician and is defined as taking ≥80% of the prescribed medicines.[1–7] It is known that adherence to long-term therapy for chronic illnesses in developed countries is only approximately 50% and adherence to topical treatments is even poorer than oral treatments.[5,8] To estimate the clinical efficacy of drugs and to examine new drugs in clinical trials, treatment adherence is of main importance. Safety, pharmacodynamics and efficacy can only be adequately assessed and interpreted if patient data on treatment adherence is available. The impact of poor adherence varies across numerous chronic skin disorders.[9,10] For instance, non-adherence to topical regimens leads to increased scores on the six area six sign atopic dermatitis (SASSAD) severity scale, indicating the disease severity in patients with atopic dermatitis.[11] For this reason, increasing adherence may even have a larger impact on patient-reported outcomes than the improvement of the treatment itself.[5]

Whereas good insight in the treatment adherence and symptoms of the patient is essential, patient-reported outcome measures are often recorded during visits and by use of paper diaries. This requires a good memory of the patient and depends on translation by the doctor / researcher which can both lead to erroneous interpretation and over or underreporting of medication use or symptoms. Paper diaries have a high recall bias, a low-to-moderate adherence rate and a limited reliability and are therefore considered as inappropriate to reliably measure treatment adherence.[12–16] Advancements in technology have enabled the widespread use of electronic diaries (e-diaries) for both the monitoring of patient outcomes and the improvement of treatment adherence in clinical trials.[13,17] In 2018, Svendsen et al. performed a randomized, controlled trial with a smartphone application for currently used topical treatment in patients with psoriasis and showed an improved short-term treatment adherence of 27% more adherence than the non-intervention group.[3]

The purpose of this study was to investigate the utility of an e-diary in 256 patients with various skin diseases participating in six clinical trials. In this study, treatment adherence and patient-reported outcomes were measured by an e-diary in six clinical trials on newly investigated topical drugs. Additionally, patient perception of usefulness and acceptability of the e-diary was evaluated.

Materials and methods

SUBJECTS AND DESIGN

From December 2014 to March 2018 six randomized, double-blind, placebo-controlled clinical trials were performed including various skin diseases. Two different topical formulations were examined in cutaneous warts (CW), atopic dermatitis (AD), genital warts (GW) and vulvar high-grade squamous intraepithelial lesions (HSIL). The Declaration of Helsinki was the guiding principle for trial execution and all subjects gave informed consent before any procedure. The studies were approved by the Dutch Medical Ethics Committee ("Stichting Beoordeling Ethiek Biomedisch Onderzoek", Assen, the Netherlands). The clinical efficacy and safety results of these studies have been or will be reported elsewhere.[18–21]

E-DIARY APPLICATION

An iOS application was developed using Xcode 7 and Objective-C according to pre-defined User Requirement Specifications and subsequently technically validated using pertaining guidelines (see supplemental Figure). The application was installed on an iPod Touch or iPhone. The patients received oral, paper and digital (in the e-diary) instructions regarding the use of the e-diary. The subjects were instructed to take pictures of the amount of the topical drug applied using the integrated camera. A maximum of four scheduled e-diary notifications were repeated every 30 minutes until the picture was taken. Subjects were instructed to apply the drug daily and to directly answer questions about patient-reported outcomes. Data was saved and securely transferred to the on-site server using encryption the following day.

TREATMENT ADHERENCE

Treatment adherence (i.e. actual administrations divided by the expected administrations) was measured by evaluating whether a patient had applied the topical drug, based on the presence of a picture in the e-diary or if absent (i.e. when for instance a technical issue occurred) after consultation of the patient. Expected entries were based on the number of patients and treatment days and calculated with the formula: number of patients times the amount of entries per day times treatment period in days.

E-DIARY ADHERENCE

E-diary adherence was positive if the e-diary was used as intended, i.e. a picture and symptom scores were entered in the e-diary for one specific day. E-diary adherence was expressed as a percentage and was measured by dividing the total number of actual entries (present pictures and/or NRS scores) by the expected entries in the entire treatment period as defined per protocol.

PATIENT-REPORTED OUTCOMES

Severity ratings of the disease or treatment-related symptoms pain and itch were assessed daily by a numeric rating scale (NRS) in the e-diary. The NRS was selected to assess pain and itch intensity once daily on a scale from 0 to 100 (0: no pain/itch and 100: worst pain/itch possible), if applicable, see Table 1. The symptom assessments were used to visualize the course of symptoms during the diseases and only patients who received placebo treatment were included in these analyses.

USER ACCEPTABILITY OF THE E-DIARY

At the end of the treatment period, all patients were asked to fill out a 14-item questionnaire (in Dutch) regarding their experience using the e-diary (Supplemental Information, questionnaire translated to English). The questionnaire consisted of multiple-choice questions and Likert-type scales regarding general user experience, technical aspects of the e-diary and adherence. Two open-ended questions allowed patients to report the strengths and weaknesses of the e-diary and to fill in any comments or suggestions.

DATA ANALYSIS

Descriptive analyses and visualization were performed using IBM SPSS (version 23, IBM Corporation, Armonk, New York, USA) and GraphPad Prism (version 6.05 for Windows, GraphPad Software, La Jolla, California, USA). Adherence was described in percentage and as the median percentage for all studies together.

Results

PATIENT CHARACTERISTICS

The use of the e-diary was evaluated in 256 patients in all treatment arms, including placebo (Table 1). The patient population in this study was the sum of patients enrolled and analyzed in the six trials, as there were no patients loss to follow up. Patients included in the trials received financial incentives.

TREATMENT AND E-DIARY ADHERENCE

The overall median treatment adherence, i.e. the proportion of patients applying the topical drug, was 98% (Table 2). This was very consistent in the different trials indicated by a narrow range of mean adherence of 97-99%.

Table 1. Clinical characteristics of patients participating in the six clinical trials. Age is shown as mean in years. Sex is described as number of patients. Treatment period is described in weeks. The e-diary was filled in during the entire treatment period.

Trial number	1	2	3	4	5	6
Trial ID (NCT)	02333643	02456480	03091426	02849262	03334240	02596074
Disease	Cutaneous warts	Atopic dermatitis	Atopic dermatitis	Genital warts	Genital warts	Vulvar HSIL
N	80	36	80	24	24	12
Age (SD)	25.8 (10.6)	24.9 (7.8)	24.4 (6.5)	34.4 (11.6)	30.8 (10.6)	49.8 (11.0)
Female	49 (61%)	27 (75%)	44 (55%)	9 (38%)	5 (20.8%)	12 (100%)
Male	31 (39%)	9 (25%)	36 (45%)	15 (63%)	19 (79.2%)	0 (0%)
Treatment	ICVT	Omiganan	Omiganan	Omiganan	ICVT	Omiganan
Dose strength	Digoxin + furosemide, digoxin, furosemide	1%, 2.5%	1%, 1.75%, 2.5%	2.5%	Digoxin + furosemide	2.5%
Active: placebo	1:1:1:1	1:1:1	1:1:1:1	2:1	3:1	2:1
Treatment period (weeks)	6	4	4	12	6	12
Regimen treatment	Once daily	Once daily	Twice daily	Once daily	Once daily	Once daily
NRS pain	-	-	-	Once daily	Once daily	Once daily
NRS itch	-	Twice daily	Twice daily	Once daily	Once daily	Once daily

NRS= numeric rating scale, ICVT= ionic contra-viral therapy, HSIL= high-grade squamous intraepithelial lesion

The median e-diary adherence, i.e. the proportion of patients capturing the applied topical drug by camera, was 93% (range 87% - 97%), see Table 3. The main reasons for not filling in the e-diary were either technical (empty device battery, no possibility of data entry after midnight) or patients forgot to take the photograph before application of the topical drug. The mean overall adherence of filling in the NRS for itch and pain was 90% for all trials together, see Table 4.

Table 2. Treatment adherence.

Trial	Expected admins[1]	Actual admins[2]	Overall treatment adherence[3]	Number of subjects with ≥80% treatment adherence
1 (CW)	3280	3187	97%	79 / 80 (99%)
2 (AD)	1013	993	98%	35 / 36 (97%)
3 (AD)	4318	4233	98%	79 / 80 (99%)
4 (GW)	1960	1942	99%	24 / 24 (100%)
5 (GW)	1008	998	99%	24 / 24 (100%)
6 (vulvar HSIL)	1020	1009	99%	12 / 12 (100%)
Overall mean	12599	12360	98%	253 / 256 (99%)
Median (range)			98% (97-99%)	100% (97-100%)

1: Expected administrations of study drugs based on number of patients and treatment days (Number of patients x treatment period in days). / 2: Actual administrations based on photographs imported via the e-diary and recall of administration asked via mail or phone. / 3: Treatment adherence is the percentage of actual admins divided by the expected admins. / CW= cutaneous warts, AD= atopic dermatitis, GW= genital warts, HSIL= high-grade squamous intraepithelial lesion

Table 3. E-diary adherence.

Trial	Expected entries[1]	Actual entries[2]	e-diary adherence[3]	Number of subjects with ≥80% e-diary adherence
1 (CW)	3280	3187	97%	79 / 80 (99%)
2 (AD)	1013	963	95%	35 / 36 (97%)
3 (AD)	4318	3958	92%	72 / 80 (90%)
4 (GW)	1960	1710	87%	17 / 24 (71%)
5 (GW)	1008	963	96%	23 / 24 (96%)
6 (vulvar HSIL)	1020	907	89%	11 / 12 (92%)
Overall mean	12599	11695	93%	237 / 256 (93%)
Median (range)			93% (87-97%)	94% (71-98%)

1: Expected entries of images in e-diary based on number of patients and treatment days (Number of patients x treatment period in days) / 2: Actual entries are the imported images of topical drug amount / 3: e-diary treatment adherence is the percentage of actual entries divided by the expected entries / CW= cutaneous warts, AD= atopic dermatitis, GW= genital warts, HSIL= high-grade squamous intraepithelial lesion

Table 4. Adherence of NRS of itch and pain. In patients with atopic dermatitis, itch was assessed twice daily.

	Itch			Pain		
Trial	Expected entries[1]	Actual entries[2]	NRS adherence[3]	Expected entries[1]	Actual entries[2]	NRS adherence[3]
2 (AD)	3192	2845	89%	N.A.	N.A.	N.A.
3 (AD)	4480	3909	87%	N.A.	N.A.	N.A.
4 (GW)	2016	1759	87%	2016	1760	87%
5 (GW)	999	962	96%	999	962	96%
6 (vulvar HSIL)	1020	957	94%	1020	957	94%
All studies	11707	10432	89%	4035	3679	91%
Median (range)	2016	1759	89% (87-96%)	1020	962	94% (87-96%)

1: Expected entries pain/itch scores based on patients and treatment days (Number of patients x treatment period in days) / 2: Actual pain/itch scores entered in the e-diary / 3: NRS pain/itch adherence is the percentage of actual entries divided by the expected entries / CW= cutaneous warts, AD= atopic dermatitis, GW= genital warts, HSIL= high-grade squamous intraepithelial lesion, N.A.= not applicable

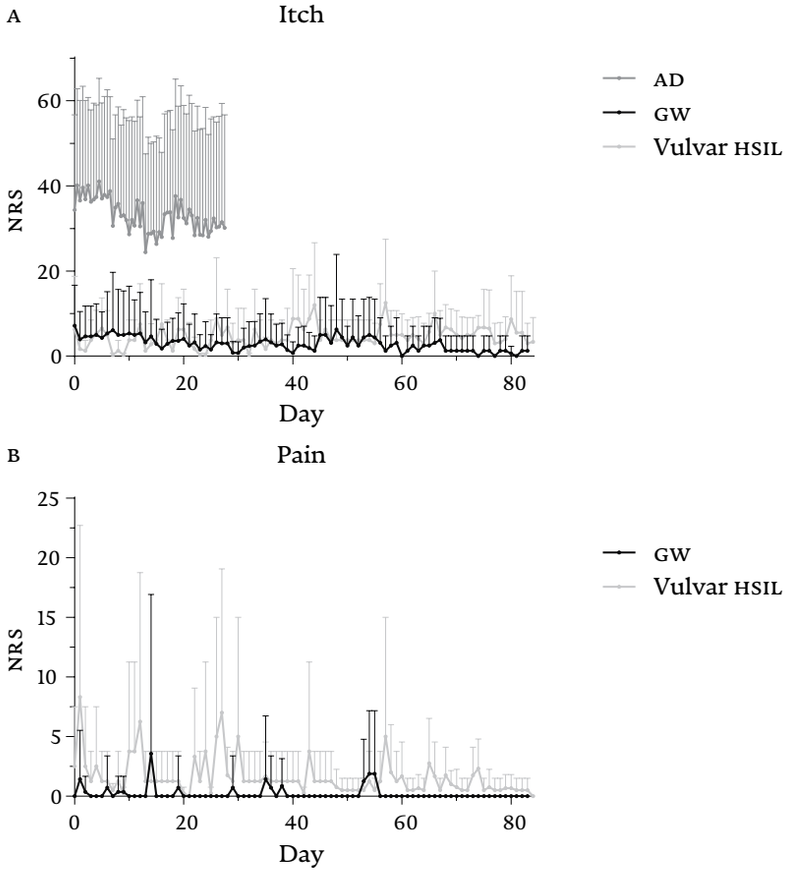
PATIENT-REPORTED OUTCOMES

Patients with AD experienced more severe itch with an higher inter-patient variability compared to patients with GW and vulvar HSIL (Fig 1A). The inter-patient variability of pain in the GW and vulvar HSIL trials was also minimal and most patients (10/14 and 2/4, respectively) experienced no pain (Fig. 1B). When examining the intra-patient variability of itch in the AD patients, there was an extensive variability in itch scores in course of disease during the 4 weeks but also between the morning and evening scores (data not shown). There was a minimal intra-patient variability of pain and itch in the GW and vulvar HSIL trials (data not shown).

USER ACCEPTABILITY OF THE E-DIARY

A total of 249 (97%) patients completed the evaluation form (Table 5). In general, the e-diary was rated good to excellent by 89% of the patients and the user-friendliness was experienced as being good to excellent by 94% of the patients. Most patients (84%) reported that it took less than 5 minutes per day to use the e-diary. Of all patients, 67% never experienced any error and 23% of the patients reported a technical problem once or twice, i.e. empty device battery. In the open-ended questions regarding the strengths and weaknesses of the e-diary, most patients commented that they found the e-diary user friendly mainly because of its simplicity. Some patients experienced problems with filling in the e-diary before midnight and also suggested to consider developing the e-diary also for android-based operating systems.

Figure 1. Symptoms itch (A) and pain (B) over time as monitored with the e-diary of patients in the placebo group. The symptoms itch and pain are monitored by using a numerical rating scale (NRS) from 0 to 100 (0 no pain/itch and 100 worst pain/itch). Per study day the mean itch of all subjects is shown +SD.



AD= atopic dermatitis (N=32), GW= genital warts (N=14), HSIL= high-grade squamous intraepithelial lesion (N=4)

Table 5. Evaluation of e-diary

General user experience		N	%
How user-friendly was the app?	Excellent	108	43%
	Good	126	51%
	Average	11	4%
	Fair	2	1%
	Poor	2	1%
In general, how would you rate the app?	Excellent	63	25%
	Good	159	64%
	Average	20	8%
	Fair	5	2%
	Poor	1	0%
How much time did it take to use the app each day?	1-5 min	209	84%
	5-10 min	37	15%
	10-15 min	2	1%
	15-20 min	0	0%
	>20 min	1	0%
How were the instructions given?	Excellent	130	52%
	Good	110	44%
	Average	9	4%
	Fair	0	0%
	Poor	0	0%
Technical aspects		**N**	**%**
How often did technical problems occur (iPod, App or Camera)?	Never	165	67%
	1-2 times	57	23%
	3-4 times	12	5%
	5-10 times	9	4%
	>10 times	5	1%
How would you rate the photo function of the app?	Excellent	67	27%
	Good	117	47%
	Average	53	21%
	Fair	8	3%
	Poor	2	1%
How would you rate the reminder-function on the app?	Excellent	46	19%
	Good	80	33%
	Average	79	32%
	Fair	39	16%
	Poor	2	1%
Did the reminder function support you to apply the gel on time?	Definitely	105	43%
	Maybe	52	21%
	No	90	36%
Adherence		**N**	**%**
How do you estimate the burden of using the app compared to a paper diary? The app is..	Much less work	146	59%
	Less work	56	23%
	Similar work	15	6%
	More work	8	3%
	Much more work	8	3%
	I do not know	13	5%
What do you prefer to use for subsequent studies?	E-diary	229	93%
	Paper diary	4	2%
	I do not know	13	5%

N= sum of all patients of all studies.

Discussion

This study is the first to show that a mobile e-diary application enhances the monitoring of patient-reported outcomes and is associated with a high treatment adherence in patients with skin disorders in an outpatient clinical trial setting. Overall, patients appreciated the e-diary and reported that the application was easy to use.

The observed treatment adherence in the current study was high compared to previously reported low adherence rates for topical treatment, i.e. up to 80% of psoriasis patients are classified as non-adherent and also adherence in atopic dermatitis patients is very poor.[5,8,22] However, before we draw convincing conclusions, there are a number of considerations that should be taken into account. At first, patients might have felt more responsible or obliged to be adherent due to a combination of our reminder strategy (i.e. patients received a second reminder when they did not correctly fill in the e-diary) and the financial incentive received. Second, we did not take the efficacy or tolerability of the drug into account, which could have influenced the adherence rate.

An additional limitation of our study is the lack of a head-to-head comparison with a paper diary. However, previous studies have already shown that paper diaries yield a much lower adherence, for instance Stone et al. found that the actual adherence of filling in pain scores with a paper diary was only 11% while adherence with an e-diary was as high as 94%.[13]

When interpreting the treatment adherence rates, it is important to additionally consider the trial protocol guidelines and their relation with real world clinical practice. The e-diary adherence in trial 4 (GW) was lowest with

87%, as patients experienced problems when applying the topical drug on a specific calendar day. As demanded by the study protocol of a well-controlled trial the time window for application was set at midnight, which was unfeasible for some patients. Therefore the time window in the study protocol in trial 5 (GW) was extended, which resulted in an improvement of e-diary adherence from 87% to 96%. The e-diary adherence in the trial involving patients with vulvar HSIL was marginally lower (89%) than in other trials, mainly caused by one subject who showed a very low treatment adherence of 30% due to not understanding the e-diary and device. It should be noted that the higher age of this population and lack of experience with mobile applications might have been a limiting factor. This is a clear indication that mobile apps do not provide a one-fits-all solution but that the use of an application needs to be carefully considered per specific age group and additional training may be required.

Altogether, we believe that our results indicate that this mobile e-diary platform can be used for the assessment of safety, efficacy and patient-reported outcomes in clinical trials in the future. We hypothesize that the reminder function of the e-diary does improve treatment adherence of patients in the six trials and can be applied to prevent under- and overdosing of topical treatments, as previously published results indicate that 67%–95% of the patients using topical treatments underdose their medication.[23,24] The e-diary will also enable the monitoring of disease-specific patient-reported outcomes and adverse events and this will support the clinician in daily clinical practice. In research settings, remote visits and monitoring could enhance recruitment and lower the burden for participants.[25] Despite of the promising features of the e-diary platform, mobile apps generally do not provide a one-fits-all solution. We should take notion of the age of future user groups, as our results also demonstrated that older patients experienced difficulties while using the application. Additional training may be required.

In conclusion, this study shows that a mobile e-diary application can be used to remotely monitor patient outcomes and treatment adherence in clinical trials with various skin disorders. Therefore, its use for personalized monitoring in the outpatient setting should be further explored. Further development of e-diaries may improve the collection of real-life patient-reported outcomes and treatment adherence, which may also lead to the improvement of disease outcomes in clinical practice.

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Chapter 3

STEREOPHOTOGRAMMETRIC 3D PHOTOGRAPHY IS AN ACCURATE AND PRECISE PLANIMETRIC METHOD FOR THE CLINICAL VISUALIZATION AND QUANTIFICATION OF HPV-INDUCED SKIN LESIONS

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Abstract

BACKGROUND Quantification of HPV-induced skin lesions is essential for the clinical assessment of the course of disease and the response to treatment. However, clinical assessments that measure dimensions of lesions using a caliper, do not provide complete insight into 3D lesions and its inter-rater variability is often poor.

OBJECTIVE The aim of this study was to validate a stereophotogrammetric 3D camera system for the quantification of HPV-induced lesions.

METHODS The camera system was validated for accuracy, precision and inter-operator and inter-rater variability. Subsequently, 3D photographs were quantified and compared to caliper measurements for clinical validation by Bland-Altman modelling, based on data from 80 patients with cutaneous warts (CW), 24 with anogenital warts (AGW) patients and 12 with high-grade squamous intraepithelial lesions of the vulva (vulvar HSIL) with a total lesion count of 220 CW, 74 AGW and 31 vulvar HSIL.

RESULTS Technical validation showed excellent accuracy (coefficients of variation (CV)≤0.68%) and reproducibility (CVs≤2%), a good to excellent agreement between operators (CVs≤8.7%) and a good to excellent agreement between different raters for all three lesion types (ICCs≥0.86). When comparing 3D with caliper measurements, excellent biases were found for diameter of AGW (long diameter 5%), good biases for diameter of AGW (short diameter 10%) and height of CW (8%) and acceptable biases were found for the diameter of CW (11%) and vulvar HSIL (short diameter 14%, long diameter 16%). An unfavorable difference between these methods (bias 25%) was found for the assessment of height of AGWs.

CONCLUSION Stereophotogrammetric 3D imaging is an accurate and reliable method for the clinical visualization and quantification of HPV-induced skin lesions.

Introduction

The human papilloma virus (HPV) is responsible for a spectrum of dermatological and gynaecological lesions. Low-risk HPV types are associated with cutaneous warts (CW) and anogenital warts (AGW), whilst high-risk types can cause (pre-)malignant lesions of the tissues of the anogenital tract, including high-grade squamous intraepithelial lesions of the vulva (vulvar HSIL, formerly referred to as usual-type vulvar intraepithelial neoplasma, uVIN).[1–4] Standard treatment for HPV-induced lesions includes surgical excision or ablative therapy; however, these can be mutilating and cause physical, psychological and (psycho-)sexual problems.[5,6] Overall, current treatment options for HPV are associated with poor response, high recurrence rates and treatment limiting side effects.[7,8] Therefore, new treatment options for HPV-related lesions are under investigation. The response to new dermatological therapies is often measured by calipers using traditional linear size assessments. These techniques fail to deliver complete insight into lesion dimensions as they can only assess the diameter and, if applicable, height of a lesion, and do not determine surface area or volumetric parameters.[9,10] Three-dimensional (3D) imaging might offer a solution to these limitations. These techniques are already widely used in the field of plastic surgery and anthropometry to add objective measuring techniques to clinical practice.[11–13] Advantages of 3D imaging include the possibility of offline 3D visualization for dimensional quantification and photo documentation over time. A validated 3D imaging system would enable the accurate and highly sensitive characterization of detailed skin lesions, and would allow for an adequate evaluation of new therapies for HPV-induced lesions.

Stereophotogrammetry is a technique that obtains two or more images from different angles, which can subsequently be reconstructed into a 3D image and has been validated for use in scars, basal cell carcinoma, wounds and wrinkles.[14–20] Currently, this method remains unvalidated for the analysis of HPV-related skin lesions. Before it can be used in the clinical practice, 3D imaging using stereophotogrammetry requires technical verification for the analysis of HPV-related lesions. Therefore, the aim of this study was to validate stereophotogrammetric 3D photography for the clinical assessment of CW, AGW and vulvar HSIL.

Materials and methods

DEVICE & ANALYSIS TOOLS

The LifeViz® Micro (Quantificare, Sophia Antipolis, France) is a compact stereophotogrammetric 3D imaging system. The package includes hardware consisting of a 15.1 megapixels, single lens reflex camera (Canon, Tokyo, Japan) with a polar flash system and a dual beam pointer used to standardize photographing distance at 20 cm. All photographs were taken according to a pre-defined standard operating procedure (see Supplemental Fig. 1) in identical environmental conditions and standardized positioning of the patient depending on lesion type (see Supplemental Fig. 2 and 3). All photographs were made in the same room with closed blinds and room lights on. The camera distance was set to "micro" to standardize the system-lesion distance to 20 cm by means of an integrated dual beam pointer. Before taking the photographs the circumstances were verified using a checklist (Supplementary Fig. 4). Photographs were always free hand images and the camera system was perpendicularly pointed at the lesion of interest. Baseline photographs were used as reference for follow-up photography. Subsequently, the photograph was taken as soon as the dual beam pointers merged onto the lesion of interest. Image management, 3D image reconstruction, and 3D analysis were performed using DermaPix® software (Quantificare, Cedex, France), which is an image management software package providing a database system and quantification features. The photographs were uploaded in the software system processed as described in Supplemental Fig. 1. The 3D analysis module was opened to generate a heat map (indicating height levels). As result 'difference' could be selected in the menu and the contour was loaded to verify the correct cement of the manual contour. The lesion dimensions were provided after loading of the contour.

TECHNICAL VALIDATION

For the technical validation of the 3D camera system, a twelve inch ruler (Schaedler Quinzel Inc., Parsippany NJ, USA) and a wart-like object manufactured out of lightweight air-drying modeling clay (Hema, the Netherlands) were used.

Accuracy of the stereophotogrammetric 3D imaging system (i.e. '3D camera system') was determined regarding absolute linear measurement by 3D photography of the twelve inch ruler and regarding multiple dimensions measurement using the wart-like object. Repeated (N=40) 3D images were taken of the wart-like phantom object under identical conditions to determine the precision of repeated measurements. The inter-operator reliability of the 3D camera system was determined to validate the use by ten different photographers. All obtained 3D images were processed and quantified by one trained assessor.

PATIENT CHARACTERISTICS

For the clinical validation, i.e. inter rater variability and clinical application, of the 3D camera system, HPV-induced skin lesions were photographed during three phase 2 clinical trials. These were randomized, double-blind, placebo-controlled studies to evaluate the efficacy and safety of a topically applied novel drug on lesions caused by HPV (Supplementary Table 1). All lesions were measured by a caliper by a trained physician and trained operators subsequently obtained 3D photographs during all study visits.

CLINICAL VALIDATION

The images of the CW were processed and quantified by four individual and independent raters. Also four individuals scored the AGW lesions. Finally, 3D images of vulvar HSIL were independently quantified by two raters. The concordance between different assessors of the 3D images in the imaging software (i.e. inter-rater variability) was determined by comparing the quantification results of all clinical 3D images of skin lesions taken at the baseline study visit.

In addition, the HPV-induced lesions in the three trials were assessed by caliper measurements and 3D photography during all visits. The 3D measurements were compared to manual measurements acquired with a digital Vernier caliper (0-150 mm, Aerospace). The caliper measurements of CW and AGW were performed by trained physicians and the vulvar HSIL measurements were performed by a trained physician and a gynaecologist. The 3D photographs were taken by trained clinical staff. For the CW and AGW, the analysis of the 3D photographs was performed after all patients completed all visits by one clinical rater. The analysis of the 3D photographs of vulvar HSIL was performed after all patients completed all visits by two raters (the trained physician and gynaecologist who also performed the caliper measurements) who independently rated all images and had a consensus meeting afterwards.

The raters who quantified the 3D photographs of the AGW and vulvar HSIL also performed the clinical caliper measurements. The quantification process was performed after all patients completed all visits. The analysis of the 3D data was performed individually and blinded to the assessment of the other operators.

The Declaration of Helsinki was the guiding principle for trial execution and all subjects gave informed consent before any procedure. The study was approved by the Dutch Medical Ethics Committee ("Stichting Beoordeling Ethiek Biomedisch Onderzoek", Assen, the Netherlands).

The clinical results on the efficacy and safety of the novel drugs investigated in the phase 2 trials will be published elsewhere.

STATISTICAL ANALYSIS

For the technical validation, the accuracy, precision and inter-operator reliability of the camera system were expressed by the mean (μ) and standard deviation (SD) per measured domain of all images, which were used to determine the coefficient of variation (CV) in percentage. We pre-specified a CV ≤5% as excellent, a CV 6-10% as good and a CV 11-20% as acceptable. The intra-class correlation coefficient (ICC) was established to quantify the variability of the 3D image processing and quantification. The ICC was determined in a two-way mixed model, with investigators as fixed and the subjects as random variable.[21] ICC values of 0.7-0.8, 0.8-0.9 and ≥0.9 were considered as of acceptable, good and excellent agreement, respectively.[22,23]

Clinical validation was approached by visualizing the data in Bland-Altman plots per parameter (where applicable; long diameter, short diameter and height) to represent the agreement between caliper and 3D measurements.[24,25] These plots calculate the mean difference between the two measurements (bias, in mm) and the limits of agreement (LOA). We used a linear mixed model specified to calculate the bias and limits of agreement based on the repeated measurements as described previously.[26] Based on the size of the lesions, we predefined the biases of ≤5%, 6-10% and 11-20% as percentages of the mean baseline caliper measurement as excellent, good and acceptable, respectively. These specifications were derived from analytical methods for clinical trials and practice.[27,28] The comparative analysis between caliper and 3D measurements was not performed until all 3D data was quantified to avoid subjectivity in the manual contours.

Results

TECHNICAL VALIDATION

The technical validation revealed a high accuracy with CVs ranging from 0 to 0.68% for 3D measurements of units of absolute ruler length (Fig. 1). The precision of a phantom object analyzed in diameter, height, volume and surface area resulted in CVs of 0.69%, 2.0%, 1.5% and 1.1%, respectively.

The inter-operator variability resulted in CVs of 1.9% for longest diameter, 8.7% for height, 2.8% for volume and 2.7% for surface area, indicating that analysis of 3D images taken by different trained persons yields similar results. For the inter-rater variability, 220 images of different CW, 72 images of different AGW and 31 different images of vulvar HSIL were quantified by the raters (see Table 1). The ICCs of the inter-rater variability for CW were 0.97, 0.90 and 0.88 for diameter, height and volume respectively. The inter-rater variability was similar for common and plantar warts (data not shown). In AGW we found an ICC of 0.91 for long diameter, 0.86 for short diameter, 0.90 for height and 0.98 for volume. For vulvar HSIL we found an ICC of 0.97 for long diameter, 0.94 for short diameter and 0.96 for surface. These data indicate that analysis of the 3D images by different raters yielded comparable results.

CLINICAL VALIDATION

Two-hundred-twenty (220) CW of 80 patients were included, of which 114 common and 106 plantar warts. In total, 1110 measurements of CW were performed, all of which (100%) were eligible for diameter, height and volume analysis. Subject characteristics are summarized in Table 1. An example of the three lesion types and the 3D reconstruction are shown in Fig 2.

Table 1. The inter-rater variability of 3D photography in HPV-induced skin lesions.

	Cutaneous warts (CW)			Anogenital warts (AGW)				Vulvar HSIL		
	Diameter	Height	Volume	Long diameter	Short diameter	Height	Volume	Long diameter	Short diameter	Surface
ICC	**0.97**	**0.90**	**0.88**	**0.91**	**0.86**	**0.90**	**0.98**	**0.97**	**0.94**	**0.96**
95% CI	0.96, 0.98	0.88, 0.92	0.85, 0.91	0.87, 0.94	0.80, 0.91	0.85, 0.94	0.96, 0.99	0.93, 0.99	0.88, 0.97	0.91, 0.98

ICC=intra-class correlation coefficient; CI=confidence interval; ICC values of >0.9 were considered as excellent and >0.8 as good

Figure 1. 3D reconstruction of the twelve inch ruler (A) and wart-like object (B). Three-D reconstruction of the twelve inch ruler by the image reconstruction software (A), and the wart-like object in a 3D reconstruction with a heat-map showing the height of the object which is used for the 3D analysis (B). *(see inside back-cover for image in color)*

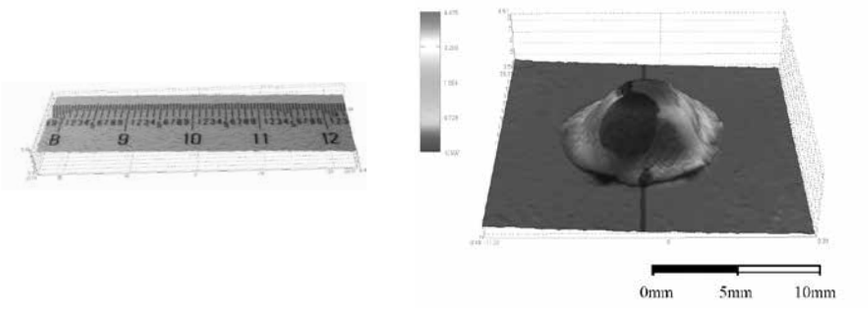


Figure 2. Three-dimensional reconstruction by stereophotogrammetry. A representative lesion for all three HPV-induced lesions (cutaneous warts, anogenital warts and vulvar HSIL) with on the left the 2D photograph, in the middle the 3D reconstruction and on the right the heat map showing height differences and the manual contour around the lesion. *(see inside back-cover for image in color)*

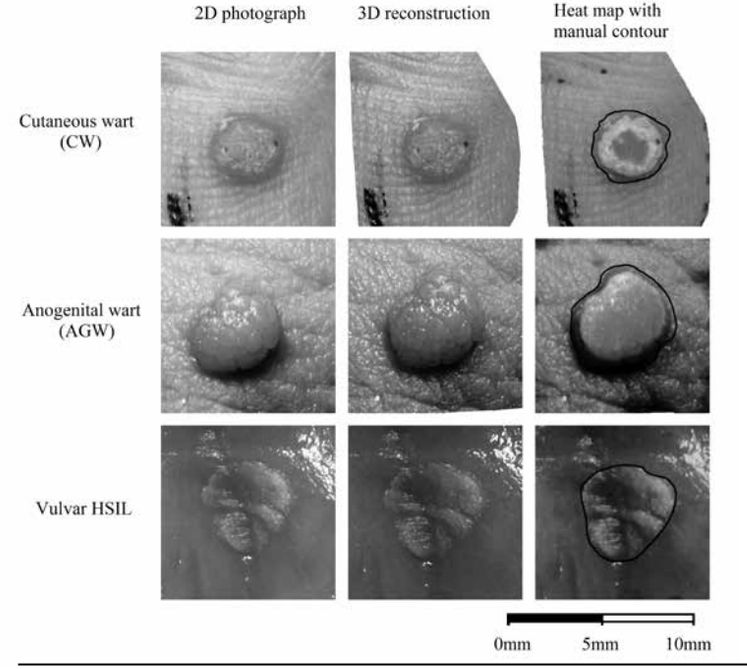
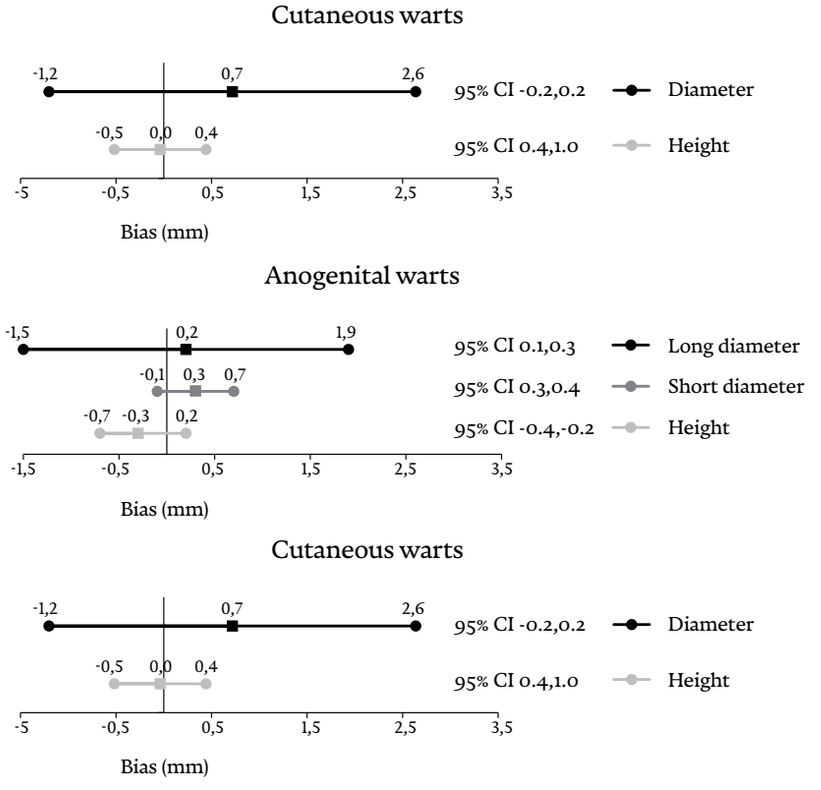


Figure 3. Forest plot of the bias and Limit of Agreement (LOA) from the Bland-Altman analysis for common and plantar warts, anogenital warts and vulvar HSIL. Forest plots of the outcomes of the Bland-Altman analysis in CW, AGW and vulvar HSIL.



CI= confidence interval, bias is indicated by a square, corresponding LoA are indicated by dots.

Forest plots of the outcomes of Bland-Altman analysis in CW, AGW and vulvar HSIL are shown in Fig. 3. In CW we found a bias of 0.7mm (11%) with LOA of -1.2mm and 2.6mm for diameter. The bias for height was -0.04mm (8%) with LOA of -0.5mm and 0.4mm (Fig. 3A). Outcomes for common and plantar warts separately showed no differences (data not shown).

Seventy-two (72) individual AGW of a total of 24 patients were included. In total, 341 measurements were performed, all of which (100%) were eligible for diameter analysis and 270 (79.2%) were eligible for height and volume

measurements. The 71 photographs (20.8%) unsuitable for height and volume measurements showed abundant presence of hairs or shadows, which impeded the construction of a 3D image. The results of the Bland-Altman analysis are shown in a forest plot (Fig. 3B). For the measurement of long diameter, we found a bias of 0.2mm (5%) with LOA between -1.5mm and 1.9mm. For short diameter we found a bias of 0.3mm (10%) and LOA ranging from -0.08mm and 0.7mm. Height bias was calculated as -0.3mm (25%) with LOA -0.7mm and 0.2mm.

Thirty-one (31) vulvar HSIL lesions of 12 patients were included and a total of 170 measurements were collected. All were eligible for diameter analysis (100%) and 164 (96.5%) were eligible for surface measurements. Photos were deemed unsuitable for further analysis when the imaging software was unable to compute a correct 3D image because of shadows and hairs disturbing the image. The bias values for long and short diameter were 2.3mm (14%) and 1.8mm (16%), with LOA ranging from -8.9mm and 13.4mm, and -7.1mm and 10.8mm, respectively (Fig. 3C).

Discussion

This validation study is the first to demonstrate that stereophotogrammetric 3D imaging is an accurate and precise method for the characterisation of HPV-related lesions and is applicable for the assessment of these lesions in a clinical setting.

The technical validation revealed an excellent accuracy with CVs<0.68%, an excellent reproducibility with CVs≤2% and a good to excellent agreement with CVs≤8.7% for the inter-operator variability. This indicates that 3D imaging is an exact and highly reproducible method. Three-dimensional photography of HPV-related lesions is also reliable for individual raters to assess the obtained photographs, established by good to excellent ICC values (0.86–0.98). These results indicate that a single photograph taken in standardized conditions by a trained operator is sufficient for reliable quantification of the lesions.

The clinical validation of diameter and height measurements yielded an acceptable (11%) and good (8%) bias between caliper and 3D imaging for CW. For AGW, we found an excellent (5%) and a good (10%) bias for the long and short diameter, respectively, but an unacceptable bias (25%) for the height of the AGW. For vulvar HSIL the calculated biases for comparison of the methods caliper and 3D measurements were acceptable for both the long (14%) and short (16%) diameter. However, we did not investigate the errors induced by variation of environmental factors such as lighting, distance, variation of the plane/positioning in an artificial manner but focused on the standardized procedure to readily use it for monitoring HPV-induced lesions in clinical practice or after intervention in a clinical trial setting. Of note, calibration of the device was not necessary as both the high accuracy (Fig. 1) and the finding of a good inter-day precision could be confirmed by the manufacturer. The latter was determined by repeatedly (N=27) analysing the geometric parameters over a period of 3 months which showed CVs ranging from 0.65% for diameter to 3.72% for volume (see Supplemental Table 2).

Our technical and clinical validation results correspond to findings in earlier studies utilizing stereophotogrammetry as a method for the qualification of dermatological lesions. Robertson and colleagues found an ICC of 0.98 for volume quantification of hemangiomas in children using the same stereophotogrammetric system as utilized in this study.[29] Moreover, a previous study evaluating pressure ulcer wounds also found excellent inter-operator variability between operators (ICC=0.99).[16] The evaluation of scars with stereophotogrammetry demonstrated excellent reliability and validity of the technique, although there was only moderate agreement between 3D quantification results and the gold standard (weighing of simulated clay scars).[14] Other studies using stereophotogrammetry for breast dimension assessments reported moderate to good agreement with manual measurements due to difficulties in exact determination of the borders.[11,12]

This study was limited by the absence of a comparison between the caliper and 3D measurements for volume and surface area. Indirectly, volume can only be calculated by a formula for an ellipse using the caliper measurements which often inaccurately reflects the shape of most warts. However, reliably including the third dimension of lesions and thus parameters like lesion volume and height of CW and AGW for detecting drug effects would be advantageous and indisputable for clinicians and drug developers. A similar limitation applies for area calculation of vulvar HSIL. There is no formula that encompasses the dimensions of HSIL and on the uneven genital tissue surface estimation techniques such as planimetry by tracing cannot be performed.[15] For these reasons, other type of research must be performed to investigate the reliability of stereophotogrammetricly acquired volume and surface estimations like the artificial system used by Skvara et all.[18]

A limitation of the imaging software was that the contour around the lesions had to be drawn manually by the investigator. A system automatically determining the borders of the lesion would be of great added value but the current auto-contour function of the software did not pass the face validity criteria, i.e. already simple assignments would lead to obvious incorrect contour representation (data not shown). However, drawing the manual outline and the subsequent automatically calculated size led to reproducible results, indicated by a low inter-rater variability (Table 1).

Limitations of stereophotogrammetry of genital HPV-related lesions concern the plane surface in the vicinity of the lesion of interest, which is a prerequisite for the analysis tool during 3D reconstruction. Achieving a plane surface is complicated in case of periungual warts, AGW on the labia, frenulum or in the perianal area, or by areas disrupted by the presence of hairs projecting over the lesion of interest. The difficulty of accurately picturing curved body parts is a feature of 3D imaging systems that has been noted previously.[13–15] These confounders might have resulted in unreliable measurement of height, surface and volume in this study. It is imperative that these data are correctly obtained by the 3D system, as caliper measurements only yield rough estimates of these parameters. For instance, we had to omit 20.8% of the volume and height quantification results of the AGW study due to suboptimal image reconstructions. Therefore, we advise potential users of stereophotogrammetry to pay extra attention to the removal of obscuring hairs during photography. We discarded images with irregularities in 3D reconstructions and erroneous measurements caused by hairs from the data that was analyzed for this study.

Recent studies suggested that following immunotherapy, lesions can firstly increase in size before regression, a phenomenon known as pseudoprogression. This is caused by influx of immune cells and measured according to the IRECIST, a guideline for response criteria for use in immunotherapeutic trials.[30,31] Future research should take into account the applicability of 3D photography as a potential biomarker for response to immune therapy in combination with histologic immune infiltration assessments.

Overall, the added value of 3D imaging over caliper measurements is the enhanced accuracy of the measurement. Additional potential applications of 3D imaging of vulvar HSIL would be collegial or post-hoc consultation, clinical follow-up and training purposes to increase disease awareness among clinicians. Furthermore, stereophotogrammetric photo documentation and offline visualization of the lesions offers an accurate and precise manner to follow the lesions during clinical trials. While the focus of our investigation was on the clinical application of 3D photography for geometric parameters of HPV-induced lesions one might speculate of its potential value for the objective measurement of lesions with distinct features such as textural changes of the skin in atopic dermatitis or other inflammatory skin conditions.

In conclusion, this study shows that stereophotogrammetry is an adequate tool for accurate and precise evaluation of HPV-induced skin lesions. Furthermore, it is applicable as a method for accurate and reproducible photo documentation of lesions. Being a portable, hand-held system, the validated system offers flexibility and practical advantages over other 3D imaging systems.[13–15] These results need validation in larger cohorts and regarding other skin conditions. In addition, future studies should aim at the examination of 3D photography as a possible biomarker for lesion size assessment and treatment response.

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SECTION II

NOVEL TOPICAL TREATMENTS FOR HPV-INDUCED DISEASES

Chapter 4

RESULTS OF PHASE 2 TRIALS EXPLORING THE SAFETY AND EFFICACY OF OMIGANAN IN PATIENTS WITH HUMAN PAPILLOMAVIRUS-INDUCED GENITAL LESIONS

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Abstract

AIMS To assess safety and tolerability and explore pharmacodynamics and efficacy of omiganan in external anogenital warts (AGW) and vulvar high-grade squamous intraepithelial lesions (HSIL).

METHODS Two randomized controlled trials in patients with external anogenital warts and vulvar HSIL were conducted. Patients received topical omiganan 2.5% or placebo gel once daily for 12 weeks with a follow-up of 12 weeks. Safety and tolerability were monitored and pharmacodynamics and clinical efficacy of omiganan were assessed by analyzing lesion count, size and viral load. Self-reported pain, itch and quality of life were assessed by an electronic diary and questionnaire.

RESULTS Twenty-four AGW and 12 vulvar HSIL patients were enrolled. All patients had a high treatment adherence (99%). No serious adverse events occurred and all adverse events (N=27) were mild, transient and self-limiting. The treatment groups were not different in terms of safety and tolerability, lesion count and size and patient-reported outcomes pain, itch and quality of life. HPV load significantly reduced after 12 weeks of treatment with omiganan compared to placebo (-96.6%; 95% CI -99.9 to – 7.4%; p=0.045) in AGW patients only.

CONCLUSIONS Topical omiganan appears to be safe in patients with AGW and vulvar HSIL and reduced HPV load after 12 weeks of treatment in AGW patients.

Introduction

Anogenital warts (AGW) and vulvar high-grade squamous intraepithelial lesion (HSIL), formerly referred to as usual type vulvar intraepithelial neoplasia (uVIN), are human papillomavirus (HPV)-induced genital lesions.[1] AGW is the most common sexually transmitted viral disease with a worldwide incidence of 160-289 per 100.000 and is mostly caused by the low-risk HPV type 6.[2–5] Vulvar HSIL is caused by high-risk HPV types, mostly type 16, and has an incidence of 1-2 per 100.000.[6–8] Vulvar HSIL has a malignant potential of 3% when treated, but up to 9% if left untreated.[6,9,10] AGW and vulvar HSIL generally cause similar symptoms such as pruritus and irritation and most patients suffer from a high psychological and sexual burden.[11–13]

The current treatment of AGW and vulvar HSIL consists of ablative/surgical and medical treatments with efficacy rates of 50-90% and high recurrence rates of 15-70%.[3,14–20] Surgical treatments are often mutilating and painful and medical treatments have notable side effects such as erythema, irritation, ulceration and pain.[12,19,20] Therefore, novel and more effective treatment options with an acceptable side effect profile are needed.
Omiganan pentahydrochloride, a synthetic analogue of indolicidin, is a small antimicrobial peptide from the cathelicidin family that has an important role in the first line immune defense of the skin and has antimicrobial properties against bacteria and fungi.[21–24] Omiganan has demonstrated to block HPV infection in vitro and has shown immuno-modulatory effects in primary human immune cell cultures.[25] The peptide modifies responses driven by cell surface-expressed toll like receptors, in particular type I and type III interferon responses which are important for efficient anti-viral activities. Omiganan also stimulates the maturation of dendritic cells, reduces the M2-type (pro-tumor) macrophage profile and enhances the M1-type (anti-tumor) macrophage profile.[25] Furthermore, omiganan is widely studied in several clinical trials in non-viral, dermatological conditions.[26–28] The combination of its antiviral and immunomodulatory properties makes topical omiganan a promising compound for the treatment of HPV-induced diseases. The main objective was to investigate the effects of topical omiganan in patients with genital dysplasia. The dose rationale was based on preclinical data where anti-HPV effects were observed at dose strength of 25 mg/g.

In this article, we report the results of two randomized controlled trials to assess safety and tolerability and to explore pharmacodynamics and clinical

efficacy of topically applied omiganan once daily in patients with AGW and vulvar HSIL. These studies were the first to test an antimicrobial peptide for its antiviral properties in HPV-induced diseases. The results of both studies are combined to establish a profile incorporating different HPV–induced diseases.

Materials and methods

STUDY DESIGN, PATIENTS AND RANDOMIZATION

Two randomized, double-blind, placebo-controlled, parallel-group, phase 2 trials in patients with AGW and vulvar HSIL were performed at the Centre for Human Drug Research (CHDR), Leiden, The Netherlands. Patients of ≥18 years with biopsy-proven HPV positive AGW or histologically proven vulvar HSIL were included. Eligible patients had at least 3 external AGW or a vulvar HSIL of 20mm in diameter or 120mm2 surface. Exclusion criteria were active treatment of the lesions within 28 days prior to enrollment, immunosuppression, pregnancy, breast-feeding or inability to use effective contraception during the treatment period and at least 90 days afterwards. All participants gave written informed consent before randomization. The studies were approved prior to any clinical activities by the independent Dutch Medical Ethics Committee (Stichting BEBO, Assen, The Netherlands).

Patients were randomly assigned to topical omiganan or placebo gel in a 2:1 ratio by a computer-generated list prepared by an independent statistician. In the vulvar HSIL trial subject numbers were sequentially allocated by chronological enrollment. In the AGW trial male patients were numbered sequentially from 1 upwards and female patients from 24 downwards in order of inclusion and were randomized in blocks of 3, to enable the inclusion of at least 3 patients of each gender per treatment. Patients, study personnel and investigators were blinded for allocated treatment throughout the study. Patients of the vulvar HSIL trial could be enrolled in an open label study part B with an additional 12 weeks treatment and 12 weeks follow-up, if there were no safety or tolerability issues in the double-blind study part and no suspected progression.

STUDY PROCEDURES

The participants were instructed by the study physician or nurse practitioner to apply the study drug once daily at approximately the same clock time for 84/85 consecutive days in the AGW and vulvar HSIL trial respectively. The amount of study drug depended on the number and size of lesions with a maximum of 37.5 mg omiganan per day. Patients returned for a visit at the study site after 2, 4, 6 (HSIL only), 8 (AGW only) and 12 weeks and 2 visits were planned during the 12-week follow-up period.

Safety and tolerability assessments were performed by monitoring of adverse events (AE), physical examination, ECG and laboratory blood and urine tests. AEs were recorded according to the Medical Dictionary for Regulatory Activities (MEDDRA) version 19. Physical examination and ECG were performed at screening and end of study visit. Blood and urine samples were collected at screening, end of treatment and end of study visit. Pharmacokinetic blood samples were taken at the last treatment day of the vulvar HSIL trial (pre-dose and 10, 20, 30, 60, 120 and 180 minutes after dosing) and tested by validated HPLC/MS/MS with a LoQ of 1.0 ng/mL of omiganan.

Clinical assessments were performed at each study visit by the same two investigators and gynecologist. In the AGW trial, three warts were selected for size measurements based on size and reproducibility of repeated measurements; target wart and biopsy wart 1 and 2. Wart size reduction was assessed in longest and shortest diameter and height (mm) was assessed using a digital Vernier caliper (0-150 mm, Conrad Electronic Benelux B.V., Oldenzaal, the Netherlands). Wart clearance was defined as complete disappearance of the wart. In vulvar HSIL patients, clinical response was determined based on the RECIST guidelines.[29] Complete response was defined as the complete disappearance of target lesions. A reduction more than 30% of the sum of the longest diameter of the target lesions was classified as partial response. Disease progression was defined as the increase of the target lesion and was expressed as the sum of longest diameters of >20%, or the development of new lesions.

Swabs were collected at each study visit by rubbing the surface of the target lesion for 5 consecutive times with a pre-wetted cotton-tipped stick. The cotton stick was placed in 1 mL of saline solution and stored at -40 degrees Celsius. Swabs for PCR analyses were taken before omiganan was applied. All swabs were analyzed batch-wise at the end of study to limit variability. Nucleic acid isolation was performed on the MagNA Pure 96 system, using the DNA and Viral NA Large Volume Kit. The input for the nucleic extraction procedure was 350µL of swab, 100µL of 0.9% NaCl and an added volume of 50µL of 10 times concentrated phosphate buffered saline. Nucleic acids were

eluted in a volume of 50µL of elution buffer. All DNA isolates were tested for PCR inhibition by a real-time PCR detecting a plasmid spiked in the PCR mix. An increase in cycle threshold value of the plasmid spike in clinical samples would indicate PCR inhibition. When PCR inhibition was found the DNA from the swab was diluted 10x and retested to confirm that PCR inhibition was no longer present. The 10x diluted sample was used in the HPV qPCR and viral load was corrected for the 10x dilution. The base line swab DNA isolates were tested on HPV genotype using the SPF10-LIPA25 version 1 (Labo Bio-medical Products B.V., Rijswijk, The Netherlands). The viral load of HPV 6 and HPV 11 in case of AGW and HPV16 in case of vulvar HSIL was determined by three separate qPCRs in swabs of lesions that were positive for the respective HPV type as determined on the baseline sample. The target of the quantitative PCRs was the E6 open reading frame and an input volume of 5µL of isolated nucleic acid was used per qPCR. Determining viral loads was done by comparing the cycle threshold value of the clinical samples with the cycle threshold values of a standard curve specific for each HPV type and analyzed in each qPCR run. Quantitative PCRs were validated and limit of detection and lower limit of quantification values for HPV6, HPV11 and HPV16 were 14/100, 12/100 and 8/32 copies/PCR respectively.

One 3mm biopsy was obtained at screening (biopsy wart 1), week 12 (biopsy wart 2) and week 24 (target wart) in case of AGW and two 3mm biopsies were obtained at screening, week 6, week 12 and week 24 of the same lesion in case of vulvar HSIL. The AGW biopsies were directly cut into two equal pieces and were further evaluated by the LUMC department of pathology. One piece/biopsy was assessed according to conventional pathological standards by the same gynecologic pathologist after hematoxylin and eosin (H&E) staining.[30,31] HPV-typing was performed in the screening biopsy using INNO-LIPA HPV genotyping Extra II (INNO-LIPA; Fujirebio Europe, Ghent, Belgium). From the other piece/biopsy RNA was isolated by means of the Nucleospin Kit RNA XS (Macherey-Nagel, Düren, Germany), cDNA was synthesized and thereafter the expression of HPV6 (AGW) or HPV16 (vulvar HSIL) was determined using real-time PCR with a primer targeted for E6.

Patient-reported outcomes were determined by a study-specific smartphone application (e-diary) and a paper questionnaire.[32] In the e-diary application, the symptoms pain and itch were assessed by a numeric rating scale (NRS) once daily during the treatment period on a scale from 0 to 100 (0: no pain/pruritus and 100: worst pain/pruritus). The paper questionnaire was filled out during the visit days and consisted of questions from a validated vulvar HSIL questionnaire (supplemental data).[33] All answers were scored and higher scores indicated a higher level of burden of disease. Scores were added up, providing mean total quality of life (QOL) scores. Higher QOL scores indicated a lower QOL. Treatment adherence, the actual administrations divided by the expected administrations, was monitored by the e-diary to register daily dose administration and to remind patients; in case patients did not fill in the e-diary, they were contacted and asked whether they applied the drug. The expression of HPV was corrected using the expression of the housekeeping genes ACTB and RPL11.

STATISTICAL ANALYSIS

Due to the exploratory nature of the trials and the first-in-indication setting, sample size was determined empirically. Safety analyses were conducted in the pre-defined intention-to-treat (ITT) population, which comprised of all enrolled patients who received at least one dose of study treatment. The pharmacodynamics and efficacy analyses were conducted in the per protocol population, which consisted of the ITT population with at least one post-baseline assessment and no major protocol deviation. All efficacy and pharmacodynamic endpoints were analyzed using a mixed model analysis of covariance (ANCOVA) using treatment, time and treatment by time as fixed factors, patient as random factor and the baseline value as covariate with SAS 9.4 for Windows (SAS institute Inc., Cary, NC, USA). A two-sided Fisher's exact and a two-sided Wilcoxon exact rank test were used to analyze wart clearance. Graphs were made using GraphPad Prism (version 6.05 for Windows, GraphPad Software, La Jolla, California, USA). All statistical tests were two-tailed with α-level of 0.05.

Results

PATIENT CHARACTERISTICS

Between November 2015 and July 2017, eligibility for participation in the AGW trial and vulvar HSIL trial was assessed in 31 and 15 individuals respectively (Figure 1). Twenty-four patients with AGW and 12 patients with HSIL were randomized. Data of one AGW patient was excluded due to a major protocol deviation (study procedure incompliance) and one AGW patient was excluded from analysis of the follow-up period due to use of concomitant treatment

for AGW (after consultancy with research physician). Seven subjects were enrolled in part B of the vulvar HSIL trial. Baseline characteristics of all patients are described in Table 1. The evaluation of HPV type showed that HPV6 (83.3%) and HPV16 (83.3%) were the most frequently occurring types in AGW and vulvar HSIL patients respectively.

Figure 1. Integrated flowchart of the AGW and vulvar HSIL trial. In the AGW trials 31 subjects were screened of whom 24 (77%) were enrolled. Of the 24 remaining subjects, 16 were randomly assigned to treatment with omiganan and 8 to placebo. One subject in the omiganan group discontinued the intervention due to personal reasons, the end of study visit was performed prematurely. One subject in the placebo group was excluded from analysis in the follow-up period because of use of concomitant medication for AGW. In the vulvar HSIL trials 15 subjects were screened of whom 12 (80%) were enrolled. Of the 12 remaining subjects 8 were randomly assigned to treatment with omiganan and 4 to placebo. All subjects completed the study.

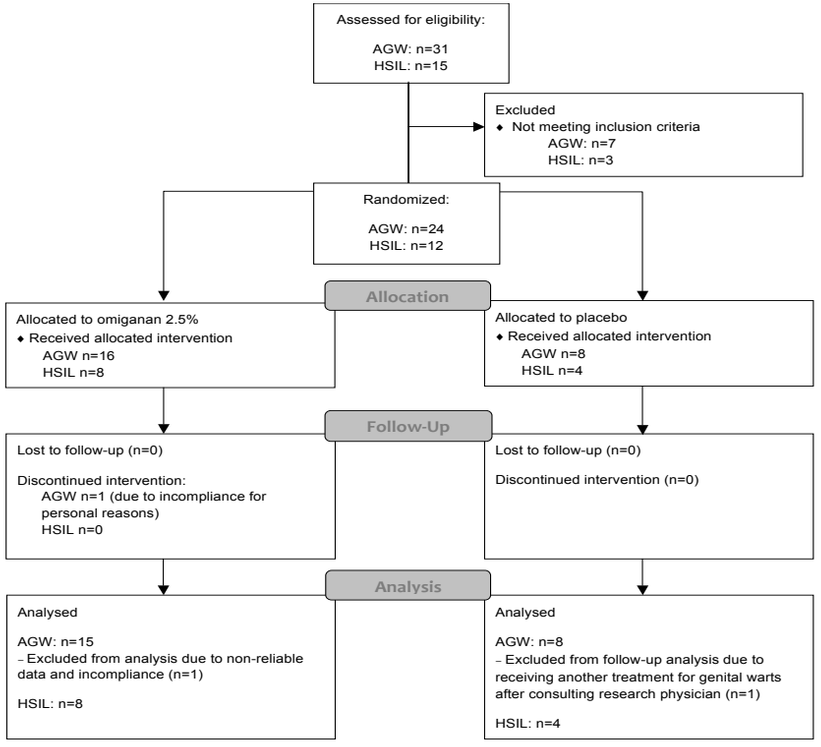

Table 1. Baseline characteristics of patients.

Characteristics	AGW trial			Vulvar HSIL trial		
	Omiganan	Placebo	Total	Omiganan	Placebo	Total
GENDER – N (%)						
Female	6 (37.5)	3 (37.5)	9 (37.5)	8 (100)	4 (100)	12 (100)
Male	10 (62.5)	5 (62.5)	15 (62.5)	-	-	-
AGE IN YEARS – *median (range)*	30 (20-46)	40 (23-64)	33 (20-64)	55 (35-71)	46 (29-51)	52 (29-71)
NUMBER OF LESIONS – *median (range)*	8 (4-62)	18 (10-43)	11 (4-62)	2 (1-3)	4.5 (2-6)	2.5 (1-6)
LESION SIZE IN MM – *median (range)*						
Long diameter	4.3 (2.4-7.8)	4.7 (4.2-7.3)	4.4 (2.4-7.8)	16 (10-40)	10 (5-30)	16 (5-40)
Short diameter	3.0 (1.4-4.8)	3.8 (2.1-5.2)	3.3 (1.4-5.2)	10 (5-20)	7 (4-30)	10 (4-30)
Height	1.3 (0.2-3.0)	1.3 (0.5-2.1)	1.3 (0.2-3.0)	-	-	-
DISEASE DURATION *In years – median (range)*	1.7 (0.3-10.5)	1.5 (0.2-5)	1.6 (0.2-10.5)	5.0 (0.1-25.8)	5.5 (0.9-8.5)	5.3 (0.1-25.8)
HPV TYPE BIOPSY - N						
HPV6	13[b]	7	20[b]	-	-	-
HPV11	-	1	1	-	-	-
HPV16	-	-	-	6[c]	4	10[c]
HPV18	-	-	-	1[c]	-	1[c]
HPV33	-	-	-	2[c]	-	2[c]
HPV44	1	-	1	-	-	-
HPV51	1[b]	-	1[b]	-	-	-
HPVX[a]	2	-	2	1	-	1
Previous treatment – N (%)	11 (69)	8 (100)	19 (79)	7 (88)	4 (100)	11 (92)
Cryotherapy	5	2	7	-	-	-
Surgical excision	3	1	4	3	1	4
Podofyllotoxine	4	7	11	-	-	-
Imiquimod	4	3	7	6	3	9
Sinecatechins	1	0	1	-	-	-
Trichloroacetic acid	1	0	1	-	-	-
Laser	-	-	-	2	1	3
Vaccination trial	-	-	-	1	0	1
SMOKING						
No	10	4	14	2	0	2
1-15/day	5	3	8	4	3	7
15+/day	1	1	2	2	1	3
SYMPTOMS						
no	13	4	17	5	3	8
itch	2	4	6	0	0	0
pain	1	0	1	0	0	0
itch and pain	0	0	0	3	1	4

a: HPVX= unidentifiable HPV type. / b: One patient had a co-infection (HPV6 and HPV51) / c: Two patients had a co-infection (HPV16 and 18, HPV16 and 33)

SAFETY, TOLERABILITY AND PHARMACOKINETICS

There were no serious adverse events and no study discontinuations or withdrawals due to adverse events. An overview of all treatment-emergent AEs is provided in Table 2. All AEs (N=34) were mild, transient and self-limiting. There were no systemic treatment-emergent adverse events. Almost all administration site symptoms (11/12) were in the omiganan group and probably related to the treatment. One AGW patient did not apply the investigational product for two weeks after having consulted the research team, due to adverse events (pruritus, erythema and irritation at application site). Hereafter, the investigational product was re-introduced and was applied every other day until the end of treatment without re-emergence of the side-effects.

Table 2. Treatment-emergent adverse events.

System organ class / preferred term	AGW				Vulvar HSIL			
	Omiganan (N=16)		Placebo (N=8)		Omiganan (N=8)		Placebo (N=4)	
	Events	Subjects	Events	Subjects	Events	Subjects	Events	Subjects
	N	N (%)	N	N (%)	N	N (%)	N	N (%)
Any event	8	7 (43.8)	1	1 (12.5)	3	3 (37.5)	0	0 (0)
ADMINISTRATION SITE CONDITIONS								
Burning sensation	1	1(6.3)	1	1(12.5)	2	2 (25)	-	-
Erythema	1	1(6.3)	-	-	-	-	-	-
Induration	1	1(6.3)	-	-	-	-	-	-
Pruritus	4	3(18.8)	-	-	1	1 (12.5)	-	-
Skin hypopigmentation	1	1(6.3)	-	-	-	-	-	-

CLINICAL EFFICACY

Clinical impressions of patients with genital warts and vulvar HSIL treated with omiganan are shown in Figure 2. The clinical efficacy of omiganan in genital warts and HSIL is summarized in Table 3. There was no difference in clearance of both AGW and vulvar HSIL between omiganan and placebo and no differences were found in study part B of the vulvar HSIL trial. A trend towards a decrease in the mean height of AGW in the omiganan group was detected compared to the placebo group (-30.3%; 95% CI -51.8 to –0.7%; p=0.054). Biopsies showed no difference in histology between the two groups of both trials, although in AGW patients treated with omiganan three biopsies were not taken because of clearance of the lesion while the in the AGW placebo group one biopsy was missing due to clearance of the lesion.

Figure 2. Photography assessments of lesions over time. Photography of a patient with angenital warts (A) and vulvar HSIL (B), both patients were treated with omiganan. Pre-dose (day 0) the lesions are clearly visible. Upon treatment, the genital warts clearly resolve (A) and the vulvar HSIL remained the same (C). The patient of picture 2a had total clearance of the genital warts at EOS, but a post-inflammatory hypopigmentation has occurred at the lesion site. Day 0 is before start of treatment, day 84 is at the end of treatment (EOT) and day 168 is at the end of study (EOS). *(see back-inside cover for image in color)*

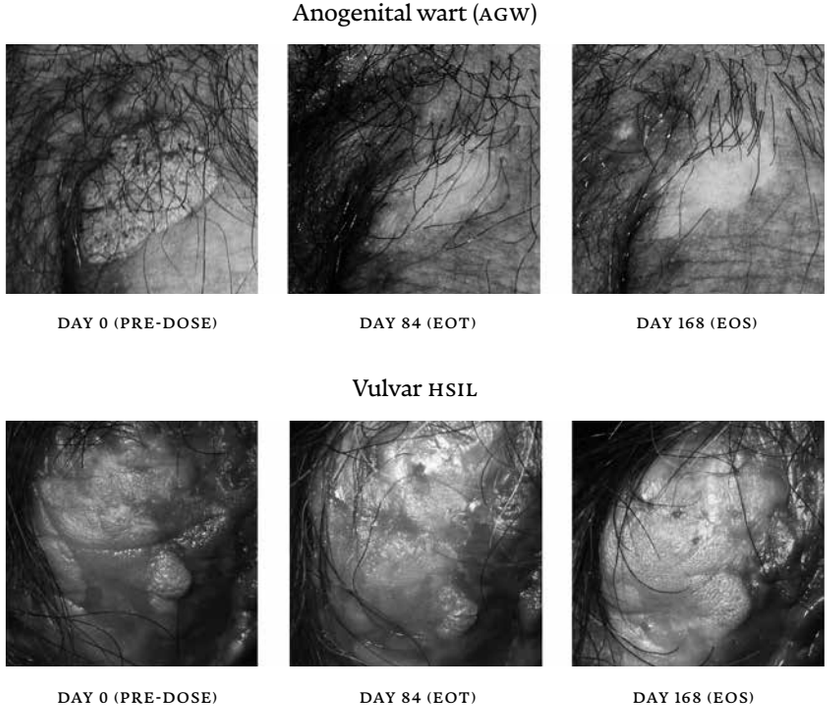

Table 3. Clinical efficacy after treatment with omiganan or placebo. Lesion count, dimensions and HPV expression are shown as mean (SD). The viral load swab is described as mean (SD) of the LN copies/microliter.

Assessment		AGW				Vulvar HSIL			
		Pre-dose	EOT	EOS	P-value	Pre-dose	EOT	EOS	P-value
Lesion count – mean (SD)	Omiganan	14.9 (14.5)	13.9 (17.2)	12.1 (15.8)	0.17	2.0 (0.9)	2.0 (0.9)	1.9 (1.0)[a]	0.58
	Placebo	21.2 (12.0)	23.5 (14.2)	18.1 (13.7)		3.8 (1.5)	3.5 (1.7)	3.3 (2.1)[a]	
Long diameter in mm – mean (SD)	Omiganan	4.4 (1.6)	4.5 (1.8)	3.8 (2.7)	0.98	-	-	-	-
	Placebo	5.0 (1.1)	6.0 (3.9)	4.8 (2.5)		-	-	-	
Short diameter in mm – mean (SD)	Omiganan	2.9 (1.0)	2.7 (1.2)	2.6 (2.0)	0.23	-	-	-	-
	Placebo	3.7 (0.9)	4.5 (2.5)	3.7 (2.0)		-	-	-	
Height in mm – mean (SD)	Omiganan	1.4 (0.8)	1.5 (1.0)	1.2 (1.1)	0.05	-	-	-	-
	Placebo	1.2 (0.6)	2.3 (2.0)	1.3 (0.7)		-	-	-	
Sum longest diameter in mm – mean (SD)	Omiganan	-	-	-	-	35.0 (17.7)	31.3 (12.0)	32.5 (11.6)	0.79
	Placebo	-	-	-		47.8 (20.7)	41.8 (22.9)	44.5 (27.6)	
Viral load swab in LN copies/µL – mean (SD)	Omiganan	5.1 (4.2)	2.3 (4.8)	1.1 (6.2)	0.045	4.8 (2.6)	5.7 (2.4)	4.8 (2.2)	0.96
	Placebo	3.6 (5.3)	4.9 (5.2)	2.5 (7.0)		7.3 (2.2)	7.4 (3.2)	7.4 (2.3)	
Relative HPV expression biopsy – mean (SD)	Omiganan	0.5 (0.8)	0.2 (0.4)	0.3 (0.3)	0.11	2.2 (1.4)	4.3 (2.8)	2.1 (1.9)	0.67
	Placebo	0.2 (0.2)	0.2 (0.2)	0.6 (0.5)		1.3 (1.1)	1.1 (1.1)	0.6 (0.6)	
Histology	Omiganan	AGW 16/16	AGW 11/15 Other 2/15 Normal 2/15	AGW 9/15 Other 3/15[b] No biopsy 3/15[c]	N.A.	HSIL 8/8	HSIL 7/8 No dysplasia 1/8	HSIL 6/8 No dysplasia 2/8	N.A.
	Placebo	AGW 8/8	AGW 6/8 Other 0/8 Normal 2/8	AGW 3/7 Other 3/7[b] No biopsy 1/7[c]		HSIL 4/4	HSIL 3/3[3]	HSIL 2/3[d] No dysplasia 1/3[d]	

EOT= end of treatment, EOS= end of study. / a At the EOT visit a biopsy of BW2 was performed / b Seborrheic verruca, (hyperkeratotic) papilloma, fibro epithelial polyp / c Due to clearance of the wart no biopsy was performed / d One patient refused the biopsies at the EOT and EOS visit

HPV CLEARANCE

The median viral load in LN copies/µL as measured with swabs at baseline was 4.6 (range -6.9 – 12.1) for AGW and 5.7 (range 1.9 – 9.5) for vulvar HSIL. In the AGW trial, the amount of viral DNA in the omiganan group showed a statistical significant decrease during the study period compared with the placebo group (-96.6%; 95% CI -99.9 to – 7.4%; p=0.045), Figure 3a. There were no differences in viral load in vulvar HSIL swabs between omiganan and placebo (Figure 3b). Remarkably, in the swabs 4 hours after treatment with omiganan a decrease was seen compared to the swabs pre-dose (-73.1%; 95% CI -94.3 – 25.9%; p=0.08). The relative expression of HPV measured in the biopsies showed no differences between omiganan and placebo in patients with AGW and HSIL (supplemental figures).

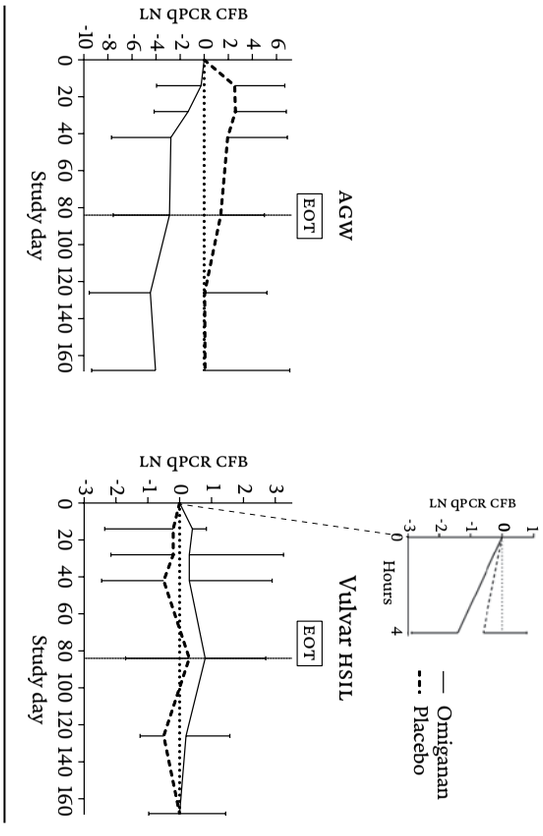

Figure 3. Viral load shown as change from baseline (CFB). A qPCR was performed to determine the HPV load in the swabs of both trials. Analysis of viral load in swabs was performed using a mixed model with treatment, time and treatment by time as fixed factors and subject as random factor. All statistical tests were two-tailed with an α-level of 0.05. A statistical significant difference was found when comparing the HPV load of AGW patients treated with omiganan compared to placebo (-96.6%; 95% CI -99.9 to – 7.4%; p=0.045) (A). No statistical difference was found when comparing the HPV load of vulvar HSIL patients treated with omiganan compared to placebo (B).

PATIENT-REPORTED OUTCOMES

For vulvar HSIL, both pain and itch showed a high inter-patient variability and no statistical significant differences were seen between the treatment groups (p=0.81 and p=0.66, in pain and itch respectively). For AGW, most patients did not experience any pain and there was a minimal inter-patient variability for both symptoms. There was no difference in total QOL score between the treatment groups in both vulvar HSIL and AGW patients (p=0.16 and p=0.94 respectively), see supplemental figures. The treatment adherence was 99% in both trials.

Discussion

This study demonstrates that despite the lack of clinical efficacy, omiganan reduced the viral load of AGW and demonstrated a favorable safety profile in AGW and HSIL patients. The distribution of the low and high-risk HPV types in AGW and vulvar HSIL patients was similar to the distribution found in literature.[5,6] This is the first study to show the use of skin swabs as a biomarker in the genital area related to antiviral treatment monitoring. We previously showed that skin swabs can be used for antiviral treatment monitoring for cutaneous warts.[35,36] These studies showed a good correlation of the HPV load between swabs and gold standard biopsies. Performing swabs instead of multiple biopsies has major advantages: a lower patient burden because of its non-invasive nature, the ability to assess the viral load of a single lesion over time and its lack of curative effect, i.e. by removing the lesion or by inducing an immune response. Previous research has shown that the viral load as assessed in cervical swabs is inversely related to HPV16 clearance and may therefore predict whether HPV infections become persistent or not.[37] A significant reduction in HPV load was found in swabs of treated AGW patients, which could suggest a suppression of the viral infection by omiganan. In the in vitro experiments we found that omiganan blocks HPV infection and has immune-modulatory effects, the reduction of the viral load in AGW patients support these in vitro results.[25] In the vulvar HSIL trial we tested the viral load 4 hours after administration of the drug and we observed a decrease of the viral load in the omiganan group. It is tempting to hypothesize that omiganan reduced the viral load shortly after application and that extended exposure (twice daily dosing) would have been more efficacious. Skin swabs from viral lesions do not represent a homogeneous sample matrix like blood. However, HPV viral load determination has previously been shown to be a reliable marker in prior studies.[36] To mitigate the risk of high variability in viral load measurements in the skin swabs a standard protocol was used by trained clinicians dedicated to the project.

We showed remarkable differences in omiganan treatment on viral load in patients with low-risk HPV type (AGW) versus patients with high-risk HPV type induced lesions (vulvar HSIL). In AGW patients, we noted a significant decrease in low-risk HPV load after omiganan treatment, opposed to no difference in high-risk HPV load in vulvar HSIL patients. While HPV6 and HPV16 are all in the Alpha genus of the phylogenetic tree of Papillomaviridae which is based on the DNA sequence of the L1 open reading frame, both HPV types exhibit different biological properties. HPV6 is mostly implicated in benign lesions whereas HPV16 is the main cause of HPV-induced cancers. In this study we investigated the effect of omiganan on two distinct lesion types. AGW is a benign lesion, whereas vulvar HSIL is a premalignant lesion, which can progress to vulvar cancer. Also, analyses of complete genome sequences have found that HPV16 is more diverse with four variant lineages, compared with two variant lineages for HPV6.[38,39] These differences along with their oncogenic properties may lead to the hypothesis that omiganan can interfere with the low-risk HPV type 6 and 11, but may not be effective for high-risk HPV type 16. A possible explanation might be that high-risk HPV types cause integration of the viral DNA into the human genome and overexpression of the E6 and E7 oncoproteins, which are not amenable to omiganan treatment.

Limitations of our study include the small sample sizes in the AGW and HSIL trials, the lack of dose ranging in both trials and the abscence of measurements of omiganan in the lesions, which makes it hard to draw conclusions about the efficacy of the drug. One patient was excluded from the efficacy analyses because of non-adherence reasons, however non-adherence was related to personal reasons, and not to treatment-related reasons or adverse events. Biopsies in AGW patients were taken from different lesions and no biopsies were taken if a lesion was cleared. It should therefore be noted that the AGW trial comprised of subjects with treatment-resistant AGW that had been present for a longer time. It cannot be excluded that omiganan might have shown higher efficacy rates in subjects with recently developed, treatment-naive AGW. Strengths of our study are the well-controlled study

designs of the AGW and HSIL trials, the use of biomarkers and innovative methodology such as the e-diary and the combination of face-to-face visits at the clinic and remote assessments by the smartphone application.

Because the reduction in viral load did not translate in clinically meaningful effects, we suggest to further develop omiganan as a potential new therapy for HPV-induced genital lesions using a higher dose (twice daily treatment) or in combination with other compounds such as imiquimod. In a human skin inflammation model study we found that omiganan can enhance the effect of imiquimod.[40] Therefore, we hypothesize that the combination therapy of omiganan and imiquimod may show synergistic effects and lower recurrence rates in patients with AGW and a follow-up study with combination treatment of omiganan and imiquimod is currently under development.

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Chapter 5

A RANDOMIZED CONTROLLED PROOF-OF-CONCEPT TRIAL OF DIGOXIN AND FUROSEMIDE IN ADULTS WITH CUTANEOUS WARTS

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Abstract

BACKGROUND Topical Ionic Contra-Viral Therapy (ICVT) comprised of digoxin and furosemide inhibits the potassium influx on which DNA viruses rely for replication. Therefore, ICVT was hypothesised to be a potential novel treatment for cutaneous warts.

OBJECTIVES To assess clinical efficacy, safety and tolerability of ICVT in adults with cutaneous warts. Secondary objective was to gain insight into underlying working mechanism of ICVT.

METHODS Treatment with ICVT was assessed for efficacy, safety and tolerability in a single- centre, randomised, double-blind, placebo-controlled phase 2A trial. Eighty adult subjects with at least 2 cutaneous warts (plantar or common) were randomised to one of 4 treatments: digoxin + furosemide (0.125%), digoxin (0.125%), furosemide (0.125%) or placebo and administered the gel once daily for 42 consecutive days. Pre-defined statistical analysis was performed with a mixed model analysis of covariance.

RESULTS Wart size and HPV load reduction was achieved in all active treatment groups. A statistically significant reduction in wart diameter of all treated warts was shown in the digoxin + furosemide treatment group versus placebo (-3.0mm; 95% CI -4.9 to -1.1mm; p=0.002). There was a statistically significant reduction in HPV load of all treated warts in the digoxin + furosemide group compared to placebo (-94%; 95% CI -100 to -19; p=0.03). With wart size reduction, histologically and immunohistochemically defined viral characteristics disappeared from partial and total responding warts.

CONCLUSIONS This study demonstrates proof-of-concept for the efficacy of topical ICVT in adults with cutaneous warts.

Introduction

Cutaneous warts, or verrucae, are a common benign skin condition with an estimated prevalence of 3-13% in the general population in the Western world.[1] Most people are affected by cutaneous warts, either plantar warts (located on foot soles), or common warts (mostly located on hands or dorsal feet), at some point in their life (Kilkenny and Marks, 1996).[1–4]

Although cutaneous warts are benign and usually resolve spontaneously, they affect both physical and psychosocial discomfort.6 Many patients use a variety of wart-removing products.[5–8] Efficacy rates of common treatments are approximately 39% for cryotherapy, 24% for salicylic acid and 46% for monochloroacetic acid, whereas spontaneous regression rates are around 16%.[7,9–11] As current treatments such as cryotherapy and monochloroacetic acid often have side effects (e.g., pain, erythema and burning sensation) and low efficacy rates, there is a need for therapies with a greater efficacy and minimal side effects.[12–15]

Cutaneous warts are caused by the human papillomavirus (HPV). The great majority (>80%) of verrucae in the general population is related to HPV1, 2, 27 and 57.[16–21]

It is well known that papillomaviruses are dependent of the milieu of the infected host cell for proliferation.[22,23] More specifically, it has been shown that DNA viruses, such as HPV rely on potassium influx (K^+) for replication.[24] The cardiac glycoside digoxin and loop diuretic furosemide both inhibit de K^+ influx by interacting with the cell membrane ion co-transporters Na^+/K^+-ATPase and Na^+-K^+-$2Cl^-$. These two compounds may therefore be valuable for the treatment of HPV-induced diseases, such as cutaneous warts. In 2006, an in vitro study found that the inhibitory effect on DNA replication was most potent when digoxin and furosemide were combined. This new approach with two well-know, established drugs, described as Ionic Contra-Viral Therapy (ICVT), is suggested to be most effective via local application.[25]

A previous phase 1/2 open-label study recently demonstrated safety and efficacy of ICVT in a group of 12 healthy subjects with common warts.[26] The aim of the current proof-of-concept study was to assess clinical efficacy, safety and tolerability of ICVT in adults with cutaneous warts in a single-center, randomised, double-blind, placebo-controlled phase 2A trial. The secondary objective was to gain insight into the underlying working mechanism of ICVT.

Materials and methods

STUDY DESIGN, PARTICIPANTS AND RANDOMIZATION

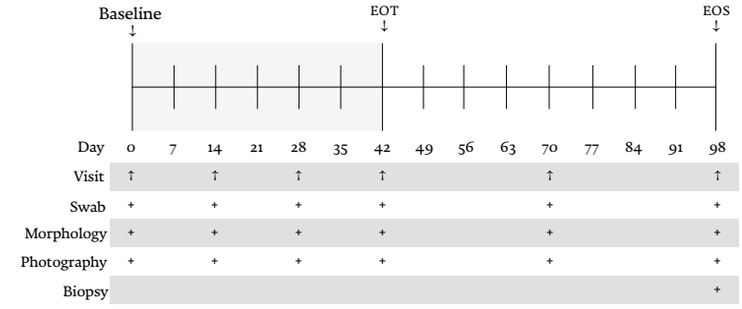
A randomised, double-blind, placebo-controlled, parallel-group, single-center phase 2 trial was conducted. The Declaration of Helsinki was the guiding principle for trial execution, and the study was approved by the independent Medical Ethics Committee "Medisch Ethische Toetsingscommissie van de Stichting Beoordeling Ethiek Biomedisch Onderzoek" (Assen, the Netherlands) prior to any procedure. Patients were included if they were (other than the skin condition) healthy, 18 years or older, and had at least 2 (non-subungual, non-genital and non-facial) common or plantar warts with a diameter of minimal 3 mm, diagnosed by a dermatologist and after giving written informed consent. A maximum of 5 warts per subtype were followed during the study. Patients were excluded if they had been exposed to wart-removing products within 30-60 days prior to enrolment, depending on the treatment. For women of childbearing age, effective contraception was required during study execution and at least 90 days afterwards. The study consisted of a screening phase (weeks -4 to 0), a treatment phase (weeks 0 to 6) and a follow-up phase (weeks 6 to 14), as shown in Figure 1.

Subjects were randomised 1:1:1:1 in blocks of four to receive one of the four treatment regimens: digoxin + furosemide (0.125%, w/w) digoxin (0.125% w/w), furosemide (0.125% w/w) or vehicle, which served as placebo with an identical appearance. Randomization was predefined and performed in SAS by an independent statistician and subject numbers were sequentially allocated by chronological enrollment. Subjects, study personnel and investigators were blinded for allocated treatment throughout the study. At baseline, all warts were numbered by a blinded independent clinical staff member; for common warts starting from 1 with a maximum of 5 and for plantar warts starting from 6 with a maximum of 10. Wart number 1 or 6 was selected as untreated wart (N=80) and the other warts were selected as treated warts. Of the treated warts 1 wart per subject was selected as primary wart (biopsy wart, N=80) using a randomly generated number in SAS drawn by an independent statistician.

STUDY SITE

The study was conducted from December 2014 to August 2015 at the Center for Human Drug Research, Leiden, The Netherlands.

Figure 1. Study design. The treatment phase entailed 42 days with study visits at day 0, 14, 28 and 42. The follow-up phase lasted for 56 days with study visits at day 70 and 98. The treatment period was 42 days with patient visits at day 0, 14, 28 and 42. At all visits the following assessments were performed of all warts: wart size measurement, wart morphology, photography, swab. At day 98, a biopsy was performed of the primary and untreated wart.



EOT = End of treatment; EOS = End of study.

STUDY PROCEDURES

The primary objective was to investigate clinical efficacy of ICVT by analyzing wart size reduction and viral load in primary warts in the four treatment groups. Wart size reduction was assessed in diameter and height (mm) by a digital vernier caliper (0-150 mm, Aerospace). Wart clearance (defined as 100% reduction) was assessed by a dermatological sub-investigator. Viral load was measured with use of skin swabs.[26] In addition, 2 biopsies of the primary wart and the untreated reference wart were taken at the end of study (EOS). The HSL-PCR/MPG assay (LMNX kit HSL-PCR, Labo Bio-medical Products) enables the simultaneous identification of 23 warts-associated HPV types from the alpha (HPV2, 3, 7, 10, 27, 28, 29, 40, 43, 57, 77, 91 and 94), gamma (HPV4, 48, 50, 60, 65, 88 and 95), mu (HPV1 and 63) and nu-genus (HPV41).[16,27] Viral load was determined for all swabs and biopsy samples of primary warts that were positive for HPV1, 2, 27 or 57 by qPCR.

The secondary objective was to gain insight into the underlying working mechanism of ICVT. Therefore, wart morphology was assessed to confirm or reject the hypothesis that wart size reduction can be predicted by morphological aspects of all warts in this study. Standardized photographs of the primary wart were taken and wart morphology was assessed using the CWARTS diagnostic tool.[28,29] Complete responders were defined as showing a reduction of 100% in size, partial responders a reduction between 25% and 100% in

size and non-responders less than 25% reduction of wart size at EOS compared to baseline. A subset of 20 warts of subjects was chosen based on response (complete, partial or non-responders) for a histopathology and immunohistochemistry (IHC) analysis, in order to confirm or reject the hypothesis that wart size reduction can be predicted by viral characteristics, Ki-67 (cell proliferation) and HPV E4 (marker of a productive infection) patterns. Viral characteristics (histopathology), Ki-67 (clone MIB-1; Dako/Agilent Technologies) and HPV E4 patterns (SILgrade-E4-1 kit containing XR-E4-1 monoclonal antibody, Labo Bio-medical Products) were assesses by two blinded reviewers and without prior knowledge of responder or HPV status. All analyses were independently performed by two reviewers except for the Ki67 analysis that was discussed during microscopy.

Safety and tolerability were monitored by tracking of adverse events (AEs), performing physical examination, measuring vital signs, 12-lead electrocardiograms, and laboratory tests (i.e. hematology, chemistry, coagulation, and urinalysis) and by systemic therapeutic drug monitoring for systemic exposure of digoxin at multiple time points throughout the study. Treatment adherence was measured by monitoring all daily dose administrations via a validated mobile e-diary application. After application of the gel, trial subjects took a photo of all warts with use of the mobile e-diary.

STATISTICS

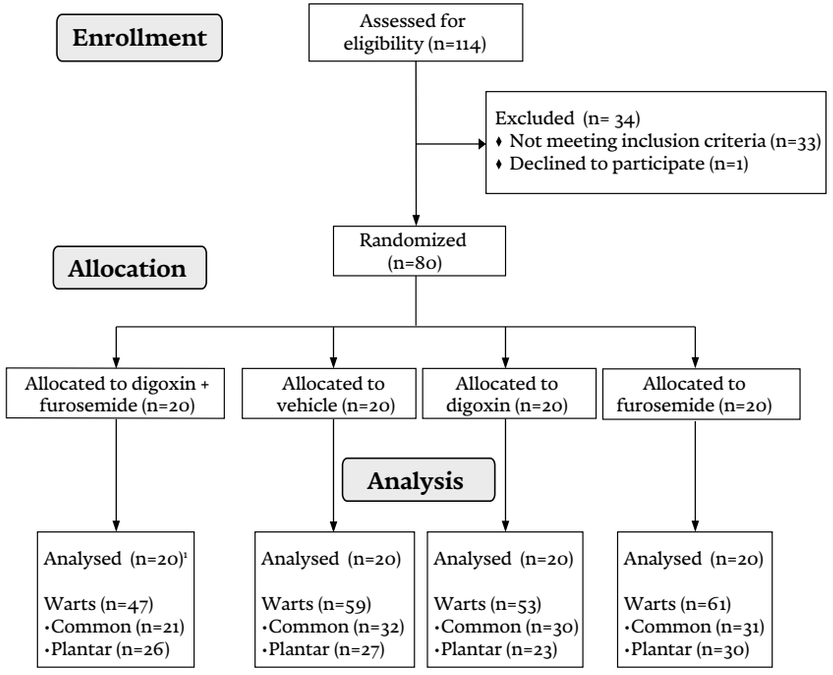
A sample size of 20 patients per treatment group was estimated based on the analysis of primary warts to provide >90% power to demonstrate superiority of digoxin and/or furosemide over placebo with a difference in means of 31.6mm^3, assuming that the common standard deviation is 30, using a two group t-test with a 0.05 two-sided significance level.[26] All efficacy/pharmacodynamic endpoints were analyzed in the intention to treat population, with a mixed model using treatment, time and treatment by time as fixed factors and subject as random factor. The pre-defined primary analyses to investigate clinical efficacy of ICVT were performed for primary warts only. For pre-defined secondary analyses to gain insight into underlying working mechanism of ICVT was based on all treated warts, and within subject was added as random factor to the model. All statistical tests were two-tailed with α-level of 0.05. A two-sided Fisher's exact and a two-sided Wilcoxon exact rank test were used to analyze wart clearance. Correlation between qPCR in swab samples and biopsies was investigated using a linear regression model with subject as random factor.

Results

PATIENTS

Hundred-and-fourteen (114) otherwise healthy subjects with cutaneous warts were screened of whom 81 (71%) were enrolled in the trial; 1 withdrew before randomization (see Fig. 1 and Fig. 2). All subjects (N=80) completed the study and there were no treatment discontinuations or early withdrawals. Baseline demographic and disease characteristics were comparable in all four treatment groups (see Table 1).

Figure 2. Flowchart of the study of all subjects and warts. One-hundred and fourteen (114) otherwise healthy subjects with cutaneous warts were screened of whom 81 (71%) were enrolled in the trial; 1 withdrew before randomization. Of the 80 remaining subjects, 20 were randomly assigned to one of four treatment groups: digoxin + furosemide, digoxin, furosemide or placebo, all to be locally applicated in gels. All subjects (N=80) completed the study and there were no treatment discontinuations or early withdrawals.



1: In the digoxin+furosemide group the pharmacodynamics measurements of the primary wart of 1 subject were excluded.

Table 1. Patient characteristics.

Characteristics	Digoxin+ Furosemide (N= 20)1	Digoxin (N= 20)	Furosemide (N= 20)	Placebo (N= 20)	Total (N=80)
Mean age in years (SD)	23.8 (±7.9)	30 (±13.5)	23.5 (±5.5)	26.1 (±12.7)	25.8 (±10.6)
SEX – NO. (%)					
Male	6 (30)	11 (55)	7 (35)	7 (35)	31 (39)
Female	14 (70)	9 (45)	13 (65)	13 (65)	49 (61)
Mean time since diagnosis in years	5.3	7.6	6.9	4.9	6.2
Total amount of warts – no.	47	53	61	59	220
Mean number of warts per subject – no.	2.4	2.7	3.1	3	2.8
Subjects with common warts – no. (%)	9 (45)	10 (50)	10 (50)	10 (50)	39 (49)
Amount of common warts – no. (%)	21 (45)	30 (57)	31 (51)	32 (54)	114 (52)
Treated common warts – no. (%)	12 (57)	19 (63)	21 (68)	21 (66)	73
Subjects with plantar warts – no. (%)	11 (55)	9 (45)	10 (50)	9 (45)	39 (49)
Amount of plantar warts – no. (%)	26 (55)	23 (43)	30 (49)	27 (46)	106
Treated plantar warts – no. (%)	15 (58)	13 (57)	20 (67)	17 (63)	65
Subjects with both common and plantar warts – no(%)	0 (0)	1 (5)	0 (0)	1 (5)	2 (3)
Size of warts – mean diameter (mm)	6.6	6.4	6.4	6.5	6.5
Size of primary wart – mean diameter (mm)	6.02	6.56	6.47	6.45	6.38
HPV TYPE PRIMARY WART					
HPV1	0	0	0	0	0
HPV2	5	4	3	6	18
HPV27	6	10	10	3	29
HPV57	6	2	3	6	17
Other[2]	2	4	4	5	15
Any previous treatment – no. (%)	16 (80)	17 (85)	14 (70)	15 (75)	62 (78)
Cryotherapy – no. (%)	12 (60)	16 (80)	12 (60)	14 (70)	54 (68)
Cimetidine – no. (%)	0 (0)	1 (5)	0 (0)	0 (0)	1 (1)
Electrocoagulation – no. (%)	0 (0)	1 (5)	0 (0)	0 (0)	1 (1)
Fluorouracil – no. (%)	0 (0)	1 (5)	0 (0)	0 (0)	1 (1)
Monochloor, Salicylic and trichloric acid – no. (%)	7 (35)	8 (40)	9 (45)	6 (30)	30 (38)
Surgery – no. (%)	1 (5)	2 (10)	0 (0)	0 (0)	3 (4)

1: In the digoxin + furosemide group the pharmacodynamics measurements of the primary wart of one subject were excluded / 2: Other = HPV3, HPV4and HPV10.

TREATMENT ADHERENCE

Seventy-eight (78) of the 80 subjects (97.5%) applied the gel once daily for more than 35 consecutive days and only sporadically subjects did not comply to the daily treatment regimen. Most subjects applied a dose within the range of 5-30 mg per wart per day. However, the mean amount of study medication applied per wart per day was highly variable (range: 2.9-118 mg).

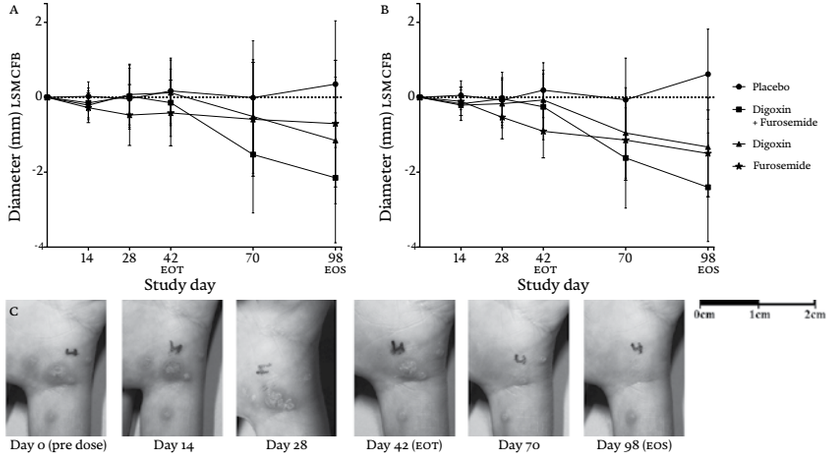
WART SIZE REDUCTION

Figure 3A shows a reduction in primary wart diameter measured by caliper from baseline to end of study (EOS) in all active treatment groups. A statistically significant effect ($p<0.05$) was found in the digoxin + furosemide group versus placebo (-2.5mm; 95% CI -4.9 to -0.1; p=0.04), while the two other treatment groups (digoxin vs placebo and furosemide vs placebo) showed no statistically significant effects (-1.5mm; 95% CI -3.9 to 0.9; p=0.21, and -1.1mm; 95% CI -3.4 to 1.3; p=0.38, respectively). Changes in diameter were most pronounced after end of treatment (EOT), as shown in Figure 3A. In the analysis of all treated warts (N=139) a statistically significant wart size reduction measured by caliper was observed between each active treatment group and placebo (digoxin + furosemide versus placebo; -3.0mm; 95% CI -4.9 to -1.1; p=0.002, digoxin vs placebo -1.9mm; 95% CI -3.7 to -0.2; p=0.03, furosemide versus placebo -2.1mm; 95% CI -3.8 to -0.4; p=0.01) as is shown in Figure 3B.

WART CLEARANCE

At the EOS, primary warts (N=80) showed comparable clearance rates in all active treatment groups, i.e. 3/19 (16%) in the digoxin + furosemide group, 3/20 (15%) in the digoxin group and 3/20 (15%) in the furosemide group. In contrast, no clearance was observed in the placebo treated group (N=20). A two-sided Fisher's exact test revealed no statistically significant differences when active treatment groups were compared to the placebo group. Table 2 shows comparable clearance rates in all treated warts in the 3 active treatment groups. Supplemental data (Table A) shows the rates of clearance observed in treated common warts (24-27%) and treated plantar warts (8-15%) at EOS. When including all warts with a reduction of ≥90% diameter, the highest response rate was seen in common warts treated with digoxin + furosemide (N=5) at EOS with a response rate of 45%. In Figure 3C an example of a photography assessment of a treated wart in the digoxin + furosemide group is shown.

Figure 3. Change from baseline (CFB) least squares mean (LSM) of diameter of primary warts (A) and all treated warts (B) and photography assessments of a common wart of subject #6 (digoxin+furosemide) (C). (A) Analysis of the primary endpoint for the intention-to-treat population (N=79) was performed using a mixed model with treatment, time and treatment by time as fixed factors and subject as random factor. All statistical tests were two-tailed with α-level of 0.05. Results showed a statistically significant reduction of wart size in the digoxin+furosemide group compared to placebo (-2.5mm; 95% CI -4.9 to -0.1; p=0.04). Single treatment groups (digoxin vs placebo and furosemide versus placebo) showed no statistically significant effects (-1.5mm; 95% CI -3.9 to 0.9; p=0.21, and -1.1mm; 95% CI -3.4 to 1.3; p=0.38, respectively). Changes in diameter were most pronounced after EOT, as shown in Figure 3A. (B) In the analysis of all treated warts (N=139) a statistically significant wart size reduction was observed between each active treatment group and placebo (digoxin+furosemide versus placebo; -3.0mm; 95% CI -4.9 to -1.1; p=0.002, digoxin vs placebo -1.9mm; 95% CI -3.7 to -0.2; p=0.03, furosemide versus placebo -2.1mm; 95% CI -3.8 to -0.4; p=0.01). (C) A photography assessment of a treated wart in the digoxin+furosemide group. *(see inside back-cover for image c in color)*



EOT= end of treatment; EOS= end of study.

Table 2. Clearance of all warts per subject at end of study.

Characteristics	Digoxin+ Furosemide (N= 191)	Digoxin (N= 20)	Furosemide (N= 20)	Placebo (N= 20)
Wart clearance[2] (p-value treatment vs placebo)	0.11	0.23	0.11	-0 (0)
All warts[3] – no. (%)	2 (11)	2 (10)	2 (10)	0 (0)
At least 1 wart, but not all warts[4] – no. (%)	1 (5)	1 (5)	2 (10)	20 (100)
No clearance – no. (%)	16 (84)	17 (85)	16 (80)	

1: In the digoxin+furosemide group the pharmacodynamics measurements of the primary wart of one subject were excluded / 2: Clearance defined as 100% reduction / 3: All warts of the subject were cleared / 4: At least one wart, but not all warts, of the subject was cleared

VIRAL LOAD

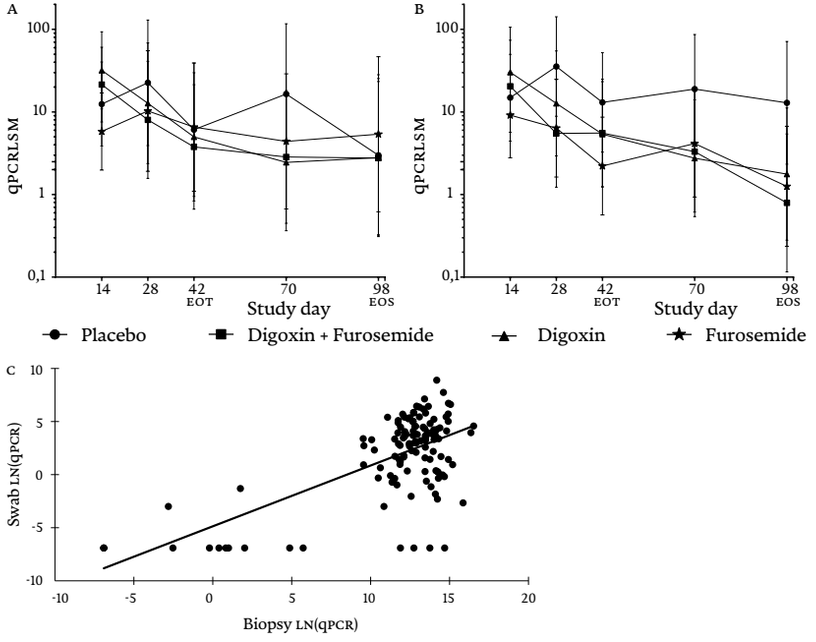
At baseline, 200 of the 219 (91%) warts (one missing sample) were positive for DNA from the 23 tested HPV types. HPV27 was most prevalent (38%) followed by HPV57 (26%) and HPV2 (24%). Of the 219 warts, 186 (85%) were positive for one of the HPV types for which viral load testing was available (i.e., HPV1, 2, 27, 57). No statistical differences were found when comparing the HPV load of primary warts (N=79) in swabs from baseline to EOS in the treatment groups with the placebo group (digoxin + furosemide -8%; 95% CI -96 to 1952; p=0.96, digoxin -6.3%; 95% CI -96 to 2086; p=0.97 and furosemide 80%; 95% CI -92 to 3966; p=0.71), as is shown in Figure 4A. However, when comparing the viral load change of HPV from baseline to EOS in the swabs of all treated warts (N=139), there was a statistically significant reduction of viral load, only in the digoxin + furosemide group versus placebo (-94%; 95% CI -100 to -19; p=0.03) (see Fig. 4B). In biopsies, no statistically significant differences in HPV load were seen in the treatment groups versus placebo. There was a significant correlation (p<0.0001) between viral load in swabs and biopsies at the EOS (Fig. 4C). We observed a significant correlation (p=0.001) between wart size reduction and reduction in HPV load (data not shown).

RESPONSE ANALYSES

In case of wart elevation, a significantly decreased wart diameter was observed at EOS after 6 weeks of treatment with the combination treatment digoxin + furosemide compared to placebo (-5.2 mm; 95% CI -8.6 to -1.8; p=0.003). The morphologic aspects callus and smooth/rough wart did not show any difference in prediction of wart size reduction (see Table 3).
In Table 4, a summary of the responder analysis is given, based on 9 responder warts (i.e., 3 complete responders, 6 partial responders) and 11 non-responders. The individual data is available in Supplemental Data: Table B. H&E staining showed changes characteristic of viral infection in biopsies from non-responder warts in contrast to the biopsies from complete and partial responder warts. In the IHC of the non-responders the Ki-67 was positive suprabasal (scattering) in all biopsies, comparing to a basal Ki-67 pattern in all complete responders, i.e. 100% and 5 out of 6 partial responders, i.e. 83.3% (Table 4). Staining of the HPV E4 protein, indicative of a productive HPV infection, was positive in all non-responders and related to a high HPV load in EOS biopsies and swabs (see Table 4). Concordantly, in all complete and partial responders

the E4 staining was negative. The mean viral load in biopsies and swabs at EOS was lower in the complete and partial responders compared to the non-responders. In Figure 5 examples of the H&E staining of a classical verruca vulgaris and plana are illustrated, showing typical viral characteristics.

Figure 4. HPV viral load in swabs depicted as percentage change from baseline (CFB) least squares mean (LSM) of primary wart (A) and all treated warts (B) and correlation of HPV viral load in swabs versus biopsy at end of study (C). (A) Analysis of primary warts (N=79) was performed using a mixed model with treatment, time and treatment by time as fixed factors and subject as random factor. All statistical tests were two-tailed with α-level of 0.05. No statistical differences were found when comparing the HPV load of primary warts (N=79) in swabs from baseline to EOS in the treatment groups with the placebo group (digoxin + furosemide -8%; 95% CI -96 to 1952; p=0.96, digoxin -6.3%; 95% CI -96 to 2086; p=0.97 and furosemide 80%; 95% CI -92 to 3966; p=0.71). (B) Viral load change of HPV from baseline to EOS in the swabs of all treated warts (N=139) was a statistically significant only in the digoxin + furosemide group versus placebo (-94%; 95% CI -100 to -19; p=0.03). (C) Correlation between qPCR in swab samples and biopsies was investigated using a linear regression model with subject as random factor. There was a significant correlation (p<0.0001) between viral load in swabs and biopsies at the EOS. The line depicts the linear correlation: Viral load swab = -4.8+0.56*viral load biopsy.



EOT= end of treatment; EOS= end of study.

SAFETY

No treatment related study discontinuations occurred. The AE profile was comparable in all treatment groups. Nasopharyngitis, headache and influenza-like illness were the most frequently occurring mild and self-limiting treatment-emergent AEs (see Supplemental Data: Table C). No clinically relevant changes in vital signs and laboratory assessments were observed. Digoxin values measured for therapeutic drug monitoring were all below the Limit of Quantification (LoQ, 300pg/mL).

Table 3. Wart morphology in relation with wart size in digoxin + furosemide treatment group.

	Wart diameter (mm)		
	Difference[1]	**95% CI[2]**	**P-value**
Callus: Present (N=42) / Absent (N=37)	-1.71	-5.12 to 1.70	0.3203
Capillary thrombosis: Present (N=45) / Absent (N=34)	-2.51	-6.15 to 1.13	0.1730
Level Elevation: (N=44) / Flat (N=35)	-5.21	-8.60 to -1.82	0.0031
Aspect Smooth: (N=17) / Rough (N=62)	-1.86	-5.85 to 2.13	0.3357

1: Difference of the mean diameter as measured by caliper / 2: CI=confidence interval

Table 4. Response analyses per responder group. HE + IHC staining biopsies and viral load in biopsies and swabs.

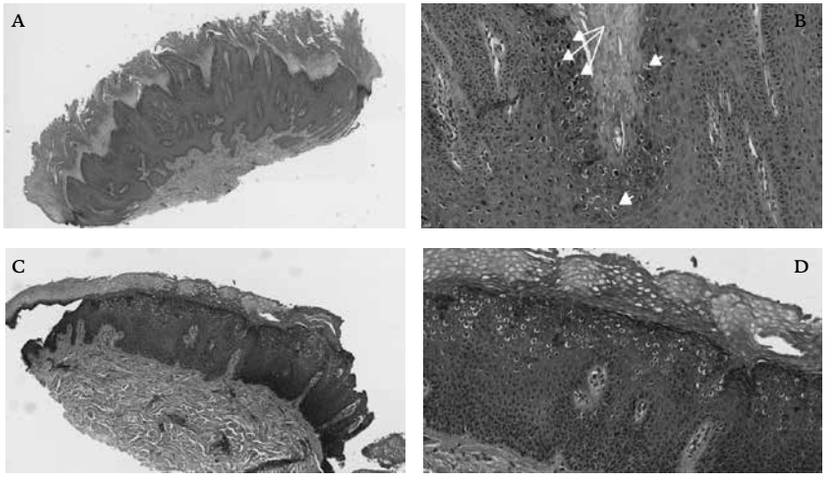
Responder	Swab baseline		Biopsy EOS		Swab EOS	
	HPV positive (n)	Mean log10 copies/PCR	Change in viral characteristics (n)	Positive test for E4, Ki-67& HPV (n)	Mean log10 copies/PCR	HPV positive (n)	Mean log10 copies/PCR
Complete (N=3)	TND[1] (1/3) HPV3 (1/3) HPV57 (1/3)	5.6[2]	0/3	E4 (0/3) Ki-67 (3/3)[3] HPV2 (1/3)	2.9[5]	HPV (0/3)	0[5]
Partial (N=6)	HPV2 (2/6) HPV27 (2/6) HPV57 (2/6)	4.7	0/6	E4 (0/6) Ki-67 (5/6)[3] (1/6)[4] HPV2 (1/6) HPV27 (1/6) HPV57 (3/6)	3.8[5]	HPV2 (2/6) HPV57 (2/6)	1.1[5,6]
Non (N=11)	HPV2 (3/11) HPV27 (4/11) HPV57 (4/11)	4.9	11/11	E4 (11/11) Ki-67 (11/11)[4] HPV2 (4/11) HPV27 (4/11) HPV57 (3/11)	8.8	HPV27 (4/11) HPV2 (4/11) HPV57 (3/11)	4.3

1: Target not detected (TND) for the 23 HPV types included in the broad spectrum genotyping assay / 2: Samples of subject without HPV DNA detected and HPV3 at baseline are not further tested for viral load and therefore not included in the mean / 3: Basal staining, restricted to the basal layer / 4: Scattered staining 5: Samples not tested are considered as zero / 6: Samples where the target is not detected are considered as zero / EOS = end of study

Discussion

This study demonstrates clear and statistically significant pharmacodynamic effects of topical ICVT on common and plantar warts with a favorable safety profile. Both lesion reduction and clearance rates indicate pharmacological activity and demonstrate proof-of-concept of ICVT in adults with cutaneous warts. Effects of ICVT was slightly more pronounced in patients with common warts. This is in accordance with previous studies wherein evident differences between response to treatment of common and plantar warts were reported.7, 30 The increased treatment resistance of plantar warts was previously described and seems to be mainly due to callus formation resulting in a decrease in cutaneous permeability of a drug.[28]

Figure 5. Histological representative cases of classical cutaneous viral warts. (A) Verruca vulgaris H&E low power view (50x) with architectural characteristic inturning of the elongated rete ridges, epidermal hyperplasia, papillomatosis, hypergranulosis, hyperkeratosis and columns of parakeratosis. (B) Verruca vulgaris H&E, detail view (200x): note koilocytes (arrowhead) and coarse granula (arrows) mostly in top layers (stratum granulosum). (C) H&E low power view (50x) of verruca plana with epidermal hyperplasia, hypergranulosis, hyperkeratosis, koilocytes in middle and upper layers. (D) verruca plana H&E, detail view (100x) note the absence of papillomatosis, parakeratosis and coarse granula. *(see inside cover for image in color)*



EFFICACY RATES THERAPIES

Efficacy rates of the most common used treatments are estimated to be around 39% for cryotherapy, 24% for salicylic acid and 46% for monochloroacetic acid. In the current study, ICVT efficacy rates were estimated to be comparable to the efficacy rates reported in literature, i.e., around 45% in common warts. However, it should be noted that the current trial consisted of subjects with treatment resistant warts that had been present for a long time (mean time of onset 4.9 – 7.6 years in the treatment groups). It can therefore be anticipated that ICVT might have shown higher efficacy rates in subjects with more recently developed warts.

DELAYED RESPONSE TO ICVT

Interestingly, wart size reduction in diameter and clearance both occurred predominantly after EOT. One explanation might be that ICVT interferes with the HPV life cycle 22, which firstly results in a reduction of HPV load and thereafter reduction in wart size. It looks like the disappearance of signs of HPV infection precedes the actual vanishing of the wart. This is supported by the fact that E4 staining, indicative of a productive infection, in the response analysis showed that partially cleared warts were in viral regression, showing less E4 signals and less papillary patterns. Another explanation could be reservoir forming of ICVT in the hyperkeratotic layer that slowly releases the drug into the lesion and thereby resulting in a delayed and prolonged response. Studies with a longer follow-up period and without the biopsy intervention at EOS have to be considered to better understand effectiveness of ICVT in both mono active and dual active form.

SYSTEMIC EFFECTS OF ICVT

Warts without application of the research gel in the active treatment groups reduced in size, in contrast to the placebo group, which suggests that this reduction was not due to spontaneous regression. The observed clearance might be explained by distant effects of the gel, i.e., increased activation of the immune system might have led to activity in untreated distant warts. Cardiac glycosides such as digoxin are known to influence the immune response at multiple levels 31, thus digoxin in the formulation might be held responsible for this. This distant clearance concept is also known from

another topical compound, imiquimod. Psoriasis patients treated with imiquimod can locally develop total body psoriasis exacerbations during treatment based on distant skin immune system activation by imiquimod.[32–34]

HPV DISTRIBUTION AND LOAD BY SWABS SUITABLE BIOMARKERS FOR CUTANEOUS WARTS

The distribution of HPV types in warts in this study was similar to the distribution found in common and plantar warts in literature 21 except for HPV1. This can logically been explained by the study sample, containing adults, whereas HPV1 infections are more prevalent among children with warts present for less than 6 months.[21]

Skin swabs have been frequently used to determine HPV status of subjects in a research setting, but not yet in relation to antiviral treatment monitoring.[35,36] Wart swabs are ideal for sampling in order to determine viral load, as the golden standard HPV status determination (biopsy) has several disadvantages such as the burden for the patient, the practical difficulty of taking multiple biopsies from a single small lesion, as well as the potential study bias caused by the curative effect of taking a biopsy.[37] The current study showed that viral load determined in swabs correlated with viral load determined from biopsies of the same wart. These data confirm the correlation previously reported by van der Kolk et al, but now in a larger sample set warranting the continued use of this marker in clinical studies.[26]

RESPONSE ANALYSES

Outcomes from microscopical and IHC analyses of the biopsies at EOS correspond with those from the viral load analysis: biopsies and swabs of the complete and partial responders have a lower viral load or are HPV negative which corresponds with loss of changes in the epithelium characteristic of viral infection, absence of E4 staining and a basal Ki-67 staining, whereas the non-responders had high viral loads in swab and biopsies. H&E staining of the biopsies showed signs of changes related to viral infection, E4 staining and a scattered Ki-67 staining. The HPV E4 protein disrupts the keratin filamentous network and inhibits formation of the cornified envelope. Detection of E4 is indicative of a productive viral infection.[22,38] Ki-67 is a biomarker for cell proliferation and in normal epithelium the Ki-67 signals are restricted to the basal layer. By reactive change, the Ki-67 positivity is also observed in the other layers of the epithelium (scattered staining).[39] From this, we can conclude that there is a clear correlation between the histopathological diagnoses, presence of E4 and Ki-67 pattern and HPV load.

Morphologic aspects of the warts could be useful to predict wart size reduction, based on the results of the current study. In clinical practice this might be helpful to have insight into the morphological characteristics when deciding about the most effective and personalised treatment.

SAFETY

Current options for therapy all have high rates of side effects including pain and irritation at the application site, blistering and scarring.[7,13] Such local irritations were not observed in the current trial.

In conclusion, our findings clearly show proof-of-concept of topical ICVT for cutaneous warts with the most pronounced effects of digoxin and furosemide when combined in a formulation for common warts. A treatment period of 42 days was well tolerated and led to significant wart size reduction and occasionally clearance. As hypothesised, wart size reduction was related to HPV load reduction measured by qPCR in swab, proving that this swab method can be a valuable, non-invasive disease biomarker for drug development in cutaneous warts. As clinical outcomes, such as clearance of lesion sites often require long-term treatment and follow-up, we indicate the found efficacy in the current study as proof-of-concept of ICVT in cutaneous warts. Further investigations to evaluate total clearance and recurrence rates after a longer treatment and follow-up period are recommended.

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Chapter 6

TOPICAL DIGOXIN AND FUROSEMIDE GEL FOR PATIENTS WITH EXTERNAL ANOGENITAL WARTS, RESULTS OF A PHASE 2 STUDY

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Abstract

BACKGROUND Anogenital warts (AGW) are caused by low-risk HPV types and represent the most common sexually transmitted viral disease. Current therapies for AGW have notable side effects and high recurrence rates. DNA viruses such as HPV rely on cellular K^+ influx. Ionic contra-viral therapy (ICVT) comprised of digoxin and furosemide inhibits the K^+ influx and is therefore a potential new treatment for AGW.

OBJECTIVES A randomized, controlled trial was performed to assess safety and tolerability and explore pharmacodynamics and clinical efficacy of ICVT in patients with AGW.

METHODS Twenty-four patients with at least 3 external AGW were randomized to either ICVT or placebo (ratio 3:1) and administered the gel once daily for 42 consecutive days. To assess safety and tolerability, laboratory safety testing was performed and adverse events, vital signs and ECGs were monitored. Clinical efficacy was assessed by lesion count and dimensions, measurement of viral load, HPV expression and histology. Patient-reported outcomes and quality of life (QOL) were assessed with use of an e-diary and paper questionnaires.

RESULTS ICVT was well tolerated as there were no clinically relevant safety findings and no serious adverse events. All adverse events (N=17) were of mild severity and self-limiting. No between-group differences in lesion count, dimensions, viral load, patient-reported outcomes and QOL were observed after treatment.

CONCLUSION ICVT is safe to be administered in patients with AGW but shows no pharmacodynamic activity or clinical efficacy after 6 weeks of treatment.

Introduction

Anogenital warts (AGW) are caused by the human papilloma virus (HPV), mostly type 6 and represent the most common sexually transmitted viral disorder.[1] AGW cause pruritus, irritation or pain and most patients experience substantial psychological burden.[2] Current treatment options are associated with low efficacy rates, serious side effects and high recurrence rates.[3,4] Therefore the development of novel effective treatments with acceptable side effects is crucial for patients with AGW. Ionic contra-viral therapy (ICVT), comprised of digoxin and furosemide, inhibits the cellular K^+ influx.[5] Recently, a phase 2 randomized-controlled trial showed a reduction in size and viral load of HPV-induced cutaneous warts after 6 weeks of treatment with topical ICVT.[6,7] Based on these findings we hypothesized that ICVT could show clinical activity in another HPV-induced disease, i.e. AGW. The objectives of this study were to evaluate safety and tolerability and to explore pharmacodynamics and clinical efficacy of ICVT in patients with AGW.

Materials and Methods

STUDY DESIGN

A randomized, double-blind, placebo-controlled, phase 2 trial was conducted from October 2017 until July 2018 at the Centre for Human Drug Research (CHDR), Leiden, The Netherlands. The study was approved by the Dutch independent Medical Ethics Committee of the Foundation BEBO (Assen, the Netherlands) prior to any procedure. Patients ≥18 years were considered eligible for the study if they had a minimum of 3 external AGW and were otherwise healthy. Patients were prohibited to use active treatment for AGW within 28 days prior to enrolment until the end of study. The patients were randomly assigned by a computer-generated list prepared by an independent statistician to either a fixed dose of a topical gel of ICVT, containing digoxin and furosemide (0.125%, w/w), or placebo in a 3:1 ratio. The study drug was applied once daily for 42 consecutive days with a follow-up period of 12 weeks. Patients, study personnel and investigators were blinded for allocated treatment throughout the study. Patients could be enrolled in an open label extension study, with 8 weeks treatment and 12 weeks follow-up, if no safety or tolerability issues occurred in the double-blind study part.

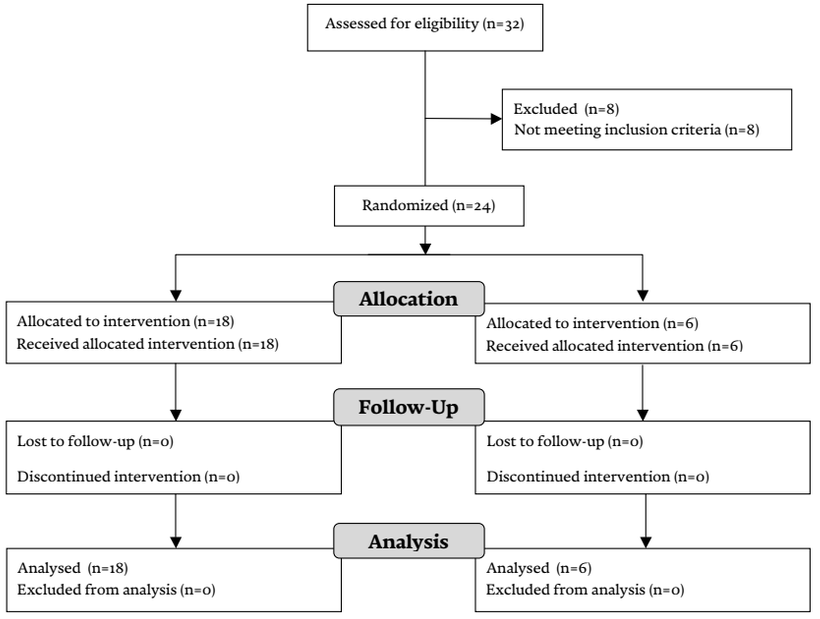
STUDY PROCEDURES

Safety and tolerability were monitored by tracking of adverse events (AEs), physical examination, vital signs, ECG and laboratory blood and urine tests. Systemic exposure of digoxin was measured during the treatment period at week 3 and 6. All warts were counted and the diameter and height (mm) of three selected warts, i.e. target wart (TW), biopsy wart 1 (BW1) and biopsy wart 2 (BW2), were measured using a digital calliper (HBM Machines B.V., Moordrecht, the Netherlands). Swabs were taken at each study visit and were analyzed in a single batch at the end of study. The HPV genotype was identified in baseline swabs using SPF10-LIPA25 version 1 (Labo Bio-medical Products B.V., Rijswijk, The Netherlands).[6–8] Viral load of HPV6 and HPV11 was determined by qPCR in all swabs. Biopsies were taken at baseline (BW1), end of treatment (BW2) and end of study (TW) and were cut in two equal pieces. One piece of the biopsy was assessed according to histopathological analyses by the LUMC department of pathology.[9,10] HPV genotyping was performed of BW1 using INNO-LIPA HPV genotyping Extra II (INNO-LIPA; Fujirebio Europe, Ghent, Belgium). From the other piece, the expression of the HPV6 E6 gene was determined using real-time quantitative reverse transcriptase PCR. Patient-reported outcomes were determined with use of an e-diary during the treatment period and quality of life (QOL) by paper questionnaires at each study visit.[11] The questionnaire was based on adapted questions from a vulvar HSIL questionnaire.[12] Treatment adherence, the actual administrations divided by the expected administrations, was monitored by the e-diary to register daily dose administration and to remind patients; in case patients did not fill in the e-diary, they were contacted and asked whether they applied the drug.

STATISTICS

Due to the exploratory nature of the trial the sample size was determined empirically. Safety analyses were conducted in the pre-defined intention-to-treat (ITT) population, comprising all enrolled patients who received at least one dose of study treatment. Pharmacodynamic and clinical efficacy analyses were conducted in the per protocol population, which consisted of the ITT population with at least one post-baseline assessment and no major protocol deviation. All efficacy and pharmacodynamic endpoints were analyzed with a mixed model analysis of covariance (ANCOVA) using treatment, time and treatment by time as fixed factors, patient as random factor and the baseline value as covariate with SAS 9.4 for Windows (SAS institute Inc., Cary, NC, USA). A two-sided Fisher's exact and a two-sided Wilcoxon exact rank test were used to analyze wart clearance. Graphs were made using GraphPad Prism (version 6.05 for Windows, GraphPad Software, La Jolla, California, USA). All statistical tests were two-tailed with α-level of 0.05.

Figure 1. Flow diagram of the study. Thirty-two subjects were screened of whom 24 (75%) were enrolled. Of the included subjects, 18 were randomly assigned to treatment with ICVT and 6 to placebo. All subjects completed the study.



Results

Twenty-four patients were enrolled and all subjects completed the trial (Fig. 1). Patient characteristics are shown in Table 1. The most frequently present HPV-type in baseline biopsy specimens was HPV6 (92%) and most patients (79%) had undergone one or more previous treatments. No serious AEs occurred and there were no study discontinuations. All AEs (N=17) were of mild severity and self-limiting. The most frequently reported AE was a burning

sensation at the application site directly after application of the gel, which was reported by 6 (33%) patients in the ICVT group and 3 (50%) patients in the placebo group. All other AEs were considered as unrelated to treatment. Safety laboratory testing, vital signs and electrocardiograms showed no differences between the treatment groups. There was no difference upon treatment in number, dimensions, viral load, HPV6 expression and histology between the ICVT and placebo group (Table 2). There was no statistical significant difference in pain scores between both treatment groups (+1.3; 95% CI -1.3 to 4.0; p=0.30). When comparing the itch scores, there was a statistically significant difference between the ICVT and placebo group (+5.9; 95% CI 0.7 to 11.2; p=0.03), because of a decrease in the mean itch score in the placebo group. There was no difference in total score of the QOL between the treatment groups (+22.4; 95% CI -70.1 to 114.9; p=0.62). Treatment adherence was 99%. Twelve subjects were enrolled in the open label extension study, which showed no differences upon treatment.

Table 1. Patient characteristics at baseline.

Characteristics	ICVT (N=18)	Placebo (N=6)	Total (N=24)
GENDER			
Female	4 (22%)	1 (17%)	5 (21%)
Male	14 (78%)	5 (83%)	19 (79%)
Age in years - median (range)	27.5 (21-44)	33 (22-67)	28 (21-67)
Number of lesions – median (range)	10 (4-51)	14 (6-19)	10 (4-51)
TARGET WART SIZE IN MM – MEDIAN (RANGE)			
Long diameter	4.6 (2.7-14.2)	6.1 (3.8-8.5)	4.8 (2.7-14.2)
Short diameter	2.8 (1.3-9.3)	3.9 (2.1-5.5)	2.8 (1.3-9.3)
Height	1.3 (0.4-9.7)	1.6 (0.8-2.3)	1.4 (0.4-9.7)
Disease duration in years – median (range)	3 (0.1-11.8)	5.4 (0.4-7.7)	3.8 (0.1-11.8)
HPV GENOTYPE BIOPSY – N (%)			
HPV6	17 (94)	5 (83)	22 (92)
HPV44	1 (6)	1 (17)	2 (4)
HPV73	0	1[1] (17)	1[1] (8)
PREVIOUS TREATMENT			
No – N (%)	3 (17)	2 (33)	5 (21)
Yes – N (%)	15 (83)	4 (67)	19 (79)
Cryotherapy	8	2	10
Surgical[2]	7	2	9
Medical[3]	13	4	17
SMOKING			
No	10	3	13
1-15/day	5	3	8
15+/ day	3	0	3
SYMPTOMS[4]			
no	10	4	14
yes	8	2	10

1: One subject had a co-infection of HPV6 and HPV73 / 2: Surgical excision and laser / 3: Podofyllotoxine, imiquimod, sinetachins, 5-FU / 4: Pain and/or pruritus / ICVT: ionic contra-viral therapy

Discussion

This study was performed to evaluate safety and tolerability and to explore pharmacodynamics and clinical efficacy of ICVT in patients with AGW. ICVT has demonstrated a favorable safety profile in patients with AGW, and that no pharmacological nor clinical activity occurred upon the once daily

Table 2. Clinical efficacy of ICVT compared to placebo.

Assessment		AGW			
		Pre-dose	EOT[1]	EOS[2]	P-value
Lesion count – mean (SD)	ICVT	15 (12.7)	15 (12.2)	9.9 (8.1)	0.89
	Placebo	12.8 (5.9)	13.3 (7.4)	6.6 (4.5)	
Long diameter in mm – mean (SD)	ICVT	5.1 (2.7)	5.5 (3.0)	4.1 (3.5)	0.49
	Placebo	6.4 (1.8)	6.5 (1.7)	5.9 (3.8)	
Short diameter in mm – mean (SD)	ICVT	3.1 (1.8)	3.5 (1.9)	2.6 (2.1)	0.84
	Placebo	3.9 (1.4)	3.7 (1.4)	3.4 (2.3)	
Height in mm – mean (SD)	ICVT	1.8 (2.2)	1.7 (2.6)	1.6 (2.9)	0.43
	Placebo	1.6 (0.6)	1.8 (1.3)	1.1 (0.7)	
Viral load swab in LN copies/µL – mean (SD)	ICVT	2.1 (5.1)	2.4 (4.8)	1.8 (4.4)	0.68
	Placebo	2.7 (4.9)	3.3 (2.1)	-0.3 (5.0)	
Relative HPV6 E6 expression biopsy – mean (SD)	ICVT	0.41 (0.05)	0.34 (0.12)	0.68 (0.08)	0.27
	Placebo	0.90 (0.06)	0.74 (0.06)	0.08 (0.001)	
Histology	ICVT	AGW 11/18 Other 7/18[3] Normal 0/18	AGW 8/18 Other 10/18[3] Normal 0/18	AGW 13/18 Other 2/18[3] Normal 0/18 No biopsy 3/18[4]	-
	Placebo	AGW 6/6 Other 0/6 Normal 0/6	AGW 3/6 Other 3/6[3] Normal 0/6	AGW 5/6 Other 1/6[3] Normal 0/6	

1: After 6 weeks of treatment / 2: After 12 weeks of follow-up / 3: Other= seborrheic verruca, fibro epithelial polyp, hyperkeratotic papilloma or reactive changes. Although, all biopsies taken pre-dose were HPV positive. / 4: Three patients refused the biopsy at the end of study / ICVT: ionic contra-viral therapy, EOT: end of treatment, EOS: end of study

administration of ICVT for 42 consecutive days. As expected, HPV6 was the most frequently present (92%) HPV-type.[13,14] In our previous trial with cutaneous wart patients ICVT showed clear reduction in wart size and viral load after 6 weeks of treatment.[15] As cutaneous warts are hyperkeratotic lesions often associated with callus growth, while AGW are commonly more smooth lesions, it is reasonable to think that the uptake and delivery of the drug in AGW is not responsible for the lack of efficacy. Two explanations can now be given for the lack of pharmacological and clinical activity of ICVT in our study. One explanation might be the difference in biological properties of the HPV type that causes AGW (HPV6) and cutaneous warts (HPV2, HPV27 and HPV57). Both are members of the Alpha-papillomavirus group.[16] In plantar warts, the HPV genotype has been found to influence natural history and treatment response.[17] Clinical practice shows that not all treatments effective in cutaneous warts are also effective in AGW, and vice versa. For example, imiquimod is registered for the use in AGW and shows an efficacy of 27-54%.[18] Several small and non-controlled trials performed to investigate the efficacy of imiquimod in cutaneous warts showed limited evidence for its efficacy.[19] A Cochrane review reported no difference in treatment with imiquimod compared to placebo, based on data from two unpublished RCTs in 391 patients with cutaneous warts.[20] On the other hand ICVT showed to inhibit other viruses such as herpes simplex and varicella zoster which makes it less plausible that the type of HPV influences this process.[5] A second explanation might be related to treatment resistance. In the current study, 79% of patients had undergone a minimum of one previous treatment for AGW and 50% had undergone 2-6 different previous treatments indicating treatment resistance. Knowing that warts were generally present for a long time (median of 3.8 years), we can therefore anticipate that ICVT could have shown slight efficacy in subjects with recently developed, treatment-naive AGW. Dose or treatment duration could also be responsible for the negative results of this trial, however these were similar to those in the previous cutaneous warts trial.

In conclusion, ICVT demonstrates to be safe to administer in patients with AGW but shows no pharmacodynamic activity or clinical efficacy after 6 weeks of treatment. The observed lack of pharmacodynamic activity of ICVT in this early-phase clinical trial, involving viral load as a relevant biomarker, facilitates further rational drug selection for AGW and might therefore compress timelines for future drug development.

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Chapter 7

SUMMARY AND DISCUSSION

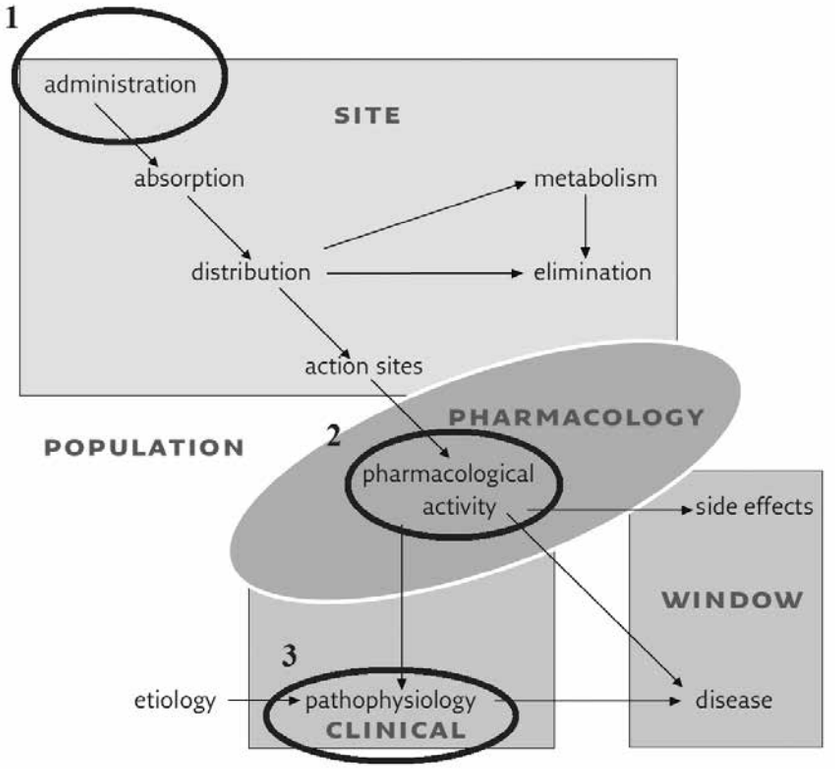
The scope of this thesis was to develop and implement new methods for the monitoring of HPV-induced disease and to elucidate novel pharmacological interventions for these disorders. The overall disease burden of HPV infections is high. As responses to current treatments are poor and recurrence rates are high, there is a strong medical need for new, effective drugs that eliminates the virus with an acceptable side effect profile. A rational, question-based development approach that integrates the investigation of the pharmacological effects in early phase drug development, will obviously be consuming less time and resources. This approach was described in 2003 in an attempt to efficiently investigate the pharmacological effects in early phase drug development.[1] In this thesis, 3 main questions of this approach were applied to clinical drug development in HPV-induced diseases as shown in Figure 1:

1. Does the drug get to the site of action, i.e. is it administrated as prescribed?
2. Does the compound cause its intended pharmacological effect?
3. Does the compound have beneficial effects on the disease?

It is essential to utilize the most appropriate methodology to answer these questions. Special attention was therefore given to the development of new methodological tools to monitor the course of HPV-related diseases in clinical trials, as well as the exploration of successful biomarkers of viral load after HPV infections. In this thesis, studies that address different aspects of early clinical phase drug development in three different HPV-related diseases are presented. This thesis is divided into two parts: section 1 describes the development and application of novel tools in clinical drug development and section 2 focuses on early phase clinical studies examining safety, tolerability, pharmacodynamic and efficacy parameters of new topical compounds with high potential for the treatment of HPV-induced diseases.

The current chapter provides a discussion of the results presented in this thesis and concerns: 1) the implementation of tools and biomarkers in early clinical trials in patients with HPV-induced disease, 2) the investigation of potential novel medical treatments of HPV-induced diseases and 3) the implications of these findings for future clinical drug development in HPV-induced diseases.

Figure 1. Schematic representation of the 3 main questions during the course of action of a drug. The first question enhances different aspects regarding reaching the site of action. We focused on one of these aspects; the administration of the drug (1) and developed an e-diary to measure the medication adherence of the patients in Chapter 2. The second question entails demonstration of the mechanism of action of the drug by showing its pharmacological activity (2) and is investigated with the use of viral load measurements in chapter 4, 5 and 6. The third question refers to the clinical efficacy which is investigated using three-dimensional photography in chapter 3. This figure is adapted from S. de Visser 2003.[1]



THE DEVELOPMENT AND IMPLEMENTATION OF TOOLS AND BIOMARKERS IN EARLY CLINICAL TRIALS IN PATIENTS WITH HPV-INDUCED DISEASE

1. DOES THE DRUG GET TO THE SITE OF ACTION?

In order to get to an answer of the first question of the question-based approach we developed an e-diary to investigate the administration of the drug by treatment adherence. Most compounds for HPV-induced diseases are topical drugs administered directly on the lesion by the patient himself at home. Importantly, medication adherence to long-term therapy is approximately 50% and adherence to topical drugs is even poorer than oral treatments.[2] **Chapter 2** described the development and implementation of the e-diary for the monitoring of treatment adherence and patient-reported outcomes in dermatological clinical trials to overcome the low adherence. The e-diary showed to be an excellent method to measure treatment administration as shown by the high treatment adherence rate (i.e. actual administrations divided by the expected administrations) of 98% (median; range 97-99%). E-diary adherence (i.e. actual entries divided by the expected entries) was also high with a median of 93% (range 87-97%) of photos capturing the applied drug. We hypothesized that this high adherence could have been the result of the designated reminder function of the e-diary to motivate patients to apply the drug on time. User acceptability of the e-diary was rated high by the patients as the e-diary was rated good to excellent by 89% of the patients and the user-friendliness was experienced as being good to excellent by 94% of the patients. Monitoring patient-reported outcomes by filling in daily symptom scores provided good insights in the disease burden. Patients filled in the itch and pain score with a median adherence of 89% (range 87-96%) and 94% (range 87-96%), respectively.

We can conclude that the e-diary seems a good tool to measure that the drug was applied on the lesions. Obviously it is not synonymous that the drug which is applied on the lesion also penetrates the skin or lesion and eventually accessing virally infected cells. However, animal models and in vitro experiments using human donor skin showed that the drugs investigated in this thesis do sufficiently penetrate the skin.[3,4] Also, the HPV-induced diseases under investigation in this thesis are all restricted to cells above the basal layer, i.e. the epidermis. This means that no transdermal drug delivery to the systemic circulation is necessary and even not desirable in case of digoxin for instance. The first question of the question-based drug development cannot be fully answered based on this research and as is shown in Figure 1 the first question entails several aspects, i.e. administration, absorption, distribution. Nevertheless, it is shown that the medication adherence is very high, implicating that the drug is administrated on the lesions and based on the preclinical studies it seems very likely that the drugs reached the sites of action.

2. DOES THE COMPOUND CAUSE ITS INTENDED PHARMACOLOGICAL EFFECT?

The second question was investigated by the measurement of the pharmacological activity of the compound. The use of a biomarker can be helpful in the early phase of clinical drug development, as it can be used as a quantitative indicator of a biological process.[5] Multiple biomarkers have already been tested for the prediction of treatment success and the prognosis in patients with HPV-induced diseases.[6–9] A useful biomarker 1) is easily applicable in clinical practice, 2) is reproducibly measurable over time, and 3) has a plausible relation between the biomarker, the expected pharmacological effects and the pathogenesis of the disease. While the drugs of concern in this thesis are hypothesized to have anti-viral effects, we choose to measure viral load of the lesions. Previous research indicated that viral load is positively related to the severity of the disease; an increase of viral load in biopsies and cytological samples of cervical lesions can predict the progression of the disease.[10,11] The gold standard to determine viral load is in biopsy samples, but these are invasive, remove (part of) the lesion and can only be performed for a limited number of times. Viral load can also be measured by taking a swab from the lesion, by rubbing the surface 5 consecutive times with a pre-wetted cotton-tipped stick. It was already shown in 2011 that swabs of cutaneous warts (CW) can reliably identify the HPV genotype.[12] In 2013, a study on genital lesions showed a high concordance between HPV genotyping by biopsy and swab in penile HSIL but only low to moderate concordance in AGW.[13] Concordance of viral load measurements in swabs and biopsy samples has never been studied in HPV-induced diseases. It is also unknown whether the viral load measured with swabs can be used to evaluate HPV-induced diseases over time, i.e. during treatment or follow-up. HPV genotyping and the determination of viral load of the lesions in HPV-induced diseases is of profound importance for the prediction of the drug efficacy and therefore may serve as a biomarker

during clinical drug development.[14] We implemented swabs to measure viral load in HPV-induced lesions over time in the trials described in **Chapter 4, 5** and **6**. Although it was found that viral load in swabs was highly variable per patient we detected significant differences in viral load over time between the different treatment groups. In Chapter 4 we evaluated viral load in biopsies and swabs of CW and found a significant correlation between both methods. Also, there was a significant correlation between the wart size reduction and the reduction in HPV load. Unfortunately, in AGW and vulvar HSIL it was not possible to compare swabs with biopsies because other laboratory techniques were used. Taking a swab is a noninvasive procedure that can be performed in the same lesion over time, but the procedure is also sensitive and has to be performed reproducibly and accurately, e.g. rubbing harder of more often could result in the collection of more viral cells. Therefore, it is important that the swab procedure is standardized and that all investigators use the same procedure. The implementation of this biomarker clarified whether the hypothesized working mechanism, i.e. anti-viral activity of the compounds, was applicable. Taken together, the measurement of viral load by taking swabs appears to be a good method to test pharmacological effects, i.e. antiviral activity, on HPV-induced lesions. With this knowledge, indications showing no anti-viral activity should not be further investigated and drug development for these specific indications should be discontinued. This approach helps saving time and resources by the early prediction of pharmacological activity and will assist in efficient drug development.

3. DOES THE COMPOUND HAVE BENEFICIAL EFFECTS ON THE DISEASE?

To determine the efficacy of a topical drug on HPV-induced lesions, it is important to frequently and precisely assess the lesions over time. For clinicians, it is often difficult to define the precise location and margins of lesions, especially in vulvar HSIL. Biomarkers can help visualizing the lesion for early detection and follow-up purposes.[15] Three-dimensional (3D) photography appears to be a good candidate biomarker to obtain comprehensive insight into the dimensions of lesions. The use of three-dimensional photography is already widely integrated into plastic surgery and anthropometry practice, but not yet applied in early phase clinical trials on drug development in HPV-induced diseases.[16–18] In clinical trials based on drug development, small changes in lesion morphology or dimensions might already predict a treatment response. We hypothesize that the 3D camera seems to be an optimal candidate biomarker of HPV-induced lesions. Therefore, the use of 3D photography was validated and investigated in **Chapter 3**. Three-dimensional photography with this specific camera system had an excellent accuracy and reproducibility. We also found a good to excellent agreement between different raters of the 3D photographs of the HPV-induced lesions. Caliper measurements of the dimensions of HPV-induced lesions are gold standard. We compared caliper and 3D measurements and found acceptable differences for the diameter of AGW, vulvar HSIL and CW and for the height of CW. Importantly, a difference between caliper and 3D measurements of the height of AGW lesions was found, probably because it was complicated to accurately measure height with a caliper of these lesions in the genital area. We speculate that 3D photography is a more reliable method than caliper-based measurements although hard evidence remains to be obtained. By using 3D photography, one might be able to determine the efficacy of a drug in an early stage of drug development. Moreover, 3D photography serves as an excellent method to clinically visualize the HPV-induced lesions as it is accurate and precise and enables researchers to compare different time points at once. The 3D camera might also be useful for the measurement of lesion surface and volume, which enables an adequate prediction of drug efficacy. We were not able to validate surface and volume measurements by the 3D camera in AGW, vulvar HSIL and CW, as these could not be measured with a caliper due to the asymmetrical and irregular shape of these lesions. Nevertheless, 3D photography seems a more suitable and versatile method to measure these lesions.

THE INVESTIGATION OF POTENTIAL NOVEL MEDICAL TREATMENTS OF HPV-INDUCED DISEASES

In the second section of this thesis two potential novel topical treatments for different HPV-induced diseases were examined: I) omiganan and II) ionic contra-viral therapy (ICVT). Omiganan is an antimicrobial peptide with immunomodulatory and anti-viral properties and was investigated in patients with AGW and vulvar HSIL as described in **Chapter 4**. Omiganan showed to be safe for both indications as there were no serious adverse events and all adverse events were of mild intensity and self-limiting. Omiganan significantly reduced viral load in AGW after 12 weeks of treatment once daily, but no clinical efficacy was shown. In vulvar HSIL, omiganan did not reduce the viral load

after 12 weeks of treatment and neither showed any clinical efficacy. ICVT is comprised of digoxin and furosemide and inhibits the potassium influx on which DNA viruses rely for replication. Safety and efficacy and in patients with CW was investigated in **Chapter 5**. ICVT was shown to be safe in patients with CW. ICVT treatment once daily for 6 weeks significantly reduced viral load and size of the CW. **Chapter 6** describes a clinical trial with ICVT in patients with AGW which showed that ICVT was well tolerated as there were no clinically relevant safety findings and no serious adverse events. Contrary to the findings in CW, ICVT in AGW patients did not show any pharmacological activity nor clinical efficacy.

DIFFERENCE IN PHARMACOLOGICAL ACTIVITY BETWEEN COMPOUNDS

It is interesting that viral load measurements showed that the compounds had varying pharmacological efficacy on the different HPV-induced lesions. Omiganan reduced viral load in AGW patients, but did not show any pharmacological activity in vulvar HSIL. ICVT showed to reduce viral load and wart size in CW, but showed no pharmacological activity in AGW. There are multiple hypotheses for these varying pharmacological effects in HPV-induced diseases.

HPV types are divided in different groups based on the alignment of the viral DNA. The HPV-induced diseases in this thesis are caused by HPV types from the Alpha genus type. Cutaneous warts are caused by low risk types HPV2, 27 and 57, AGW mostly by low risk type HPV6, while vulvar HSIL is mostly caused by the high risk type HPV16. The phylogenetic tree of HPV (see Figure 3 in the introduction of this thesis) shows that these types are evolutionary different.[19] Previous research indicated that the HPV type can predict treatment response in patients with CW.[20] It was found that HPV2 and HPV27 were associated with a limited response to the treatment of plantar warts with monochloroacetic acid or the combination of cryotherapy and salicylic acid. HPV1 can be a predictor of the response to the treatment of plantar warts with the combination of cryotherapy and salicylic acid. It is plausible that the difference in treatment response was caused by the difference in the causative HPV type.

We tested omiganan in AGW and vulvar HSIL. In AGW, treatment with omiganan reduced viral load, while in vulvar HSIL no effect was observed. AGW is a benign lesion caused by low risk HPV types, whereas vulvar HSIL is a premalignant lesion causes by high risk HPV types and can progress to vulvar cancer. Analyses of complete genome sequences have shown that HPV16 is more diverse with four variant lineages, compared to two variant lineages for HPV6.[21,22] These differences and their oncogenic properties may point towards the hypothesis that omiganan can interfere with the low-risk HPV type 6 and 11, but may not be effective for high-risk HPV type 16. An explanation might be that high risk HPV types cause integration of the viral DNA into the human genome and overexpression of the E6 and E7 oncoproteins, which are not amenable to omiganan treatment.

The location and lesion type might also be of importance when predicting treatment response, e.g. penetration of drug might be easier on the mucosa instead of on a cutaneous wart.

THE IMPLICATIONS OF THESE FINDINGS FOR FUTURE CLINICAL DRUG DEVELOPMENT IN HPV-INDUCED DISEASES

The question-based drug development approach is useful for the design and conduct of (early phase) clinical trials in HPV-induced diseases. As shown in this thesis, the e-diary is an adequate tool to measure treatment adherence and patient-reported outcomes. In addition to that, the e-diary could also stimulate timely use of medication. In future clinical trials, patient-reported outcomes need further investigation. Also, the e-diary might be highly valuable to adjust the treatment based on patient-reported outcomes in clinical practice. Integration of e-diaries in clinical practice is gaining increased attention, for example in COPD patients where electronic questionnaires are developed to predict symptom-defined exacerbations.[23]

Viral load measurement by using swabs is a suitable biomarker for the prediction of the anti-viral pharmacological efficacy of the drug. Viral load in swabs and biopsies had a good correlation in CW patients and therefore the swabs can replace the biopsies for the measurement of viral load in CW. The comparison of the viral load of swabs and biopsies in AGW and vulvar HSIL needs to be further investigated in future studies. For future implementation of 3D photography, validation of surface and volume measurements is warranted and another system might be necessary for the irregular shapes of the genital area.

We encountered some difficulties with the precise measurement of the vulvar HSIL dimensions with both caliper and 3D photography. Vulvar HSIL

is associated with a highly variable appearance and most of the time lesions have irregular and blurred borders and are difficult to recognize. In future drug development initiatives it would therefore be ideal to improve the visualization of these HPV-induced lesions, for example with a specific fluorescent ligand for HPV-induced lesions. Fluorescence imaging has well-known advantages in intraoperative settings. Particularly near-infrared (NIR) fluorescence seems interesting to investigate HPV-induced lesions, as its light increases tissue penetration depth up to 1 cm. A fluorescent agent can either be nonspecific or targeted at HPV positive cells for example. NIR fluorescence has already been widely investigated in cancer surgery for other indications, such as ovarian and colorectal cancer.[24,25] Imaging using NIR fluorescence should be further investigated in HPV-induced diseases and will hopefully facilitate the measurement of vulvar HSIL and other lesions in early stage clinical research. In addition, NIR fluorescence can also be valuable in clinical practice for lesion diagnosis and visualization during operations, to ensure total lesion removal.

In conclusion, in this thesis we implemented the question-based drug development approach in clinical trials in HPV-induced diseases. This led to the implementation of 1) the e-diary to confirm whether the drug has reached the site of action, 2) viral load measurement by swabs to determine the pharmacological activity of the compound and 3) three-dimensional photography to investigate the efficacy of the compound. Together, the described tools and biomarkers might be of high value for a more efficient drug development in HPV-induced diseases. Also, four clinical trials were performed with the topical drug omiganan and ICVT in patients with different HPV-induced diseases. These trials showed that both drug have different pharmacological and clinical efficacy depending on the HPV-induced disease.

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Chapter 8

NEDERLANDSE SAMENVATTING

Dit proefschrift beschrijft een aantal klinische studies over aandoeningen veroorzaakt door het humaan papillomavirus (HPV). Deze studies gaan ten eerste over nieuwe instrumenten om de effectiviteit van geneesmiddelen te onderzoeken en ten tweede over twee nieuwe geneesmiddelen, die zijn getest in verschillende HPV-geïnduceerde ziektebeelden. Nieuwe behandelingen voor deze ziekten zijn noodzakelijk omdat de huidige behandelingen vaak onvoldoende werken en/of vervelende bijwerkingen hebben. Daarnaast zijn de beschikbare behandelingen geassocieerd met een hoge kans op terugkeer van de ziekte.

HPV is een virus dat de epitheelcellen van de huid en slijmvliezen kan infecteren. Dit is in de meeste gevallen een asymptomatische infectie, maar een persisterende (aanhoudende) infectie kan verschillende aandoeningen veroorzaken. De genetische samenstelling van HPV kan worden onderverdeeld in vroege (E1-E2, E4-E7) en late genen (L1-L2). De vroege genen zijn betrokken bij het tot stand komen van de infectie en de late genen bij de verspreiding van het virus. De verschillende types HPV worden onderverdeeld in 5 groepen: Alfa, Bèta, Gamma, Mu en Nu. De Alfa groep is de grootste en omvat de seksueel overdraagbare types. HPV kan ook worden getypeerd als een laag-risico of hoog-risico virus, dat respectievelijk goedaardige aandoeningen of (voorstadia van) kanker kan veroorzaken. In dit proefschrift worden 3 soorten door HPV geïnduceerde ziekten onderzocht: huidwratten, genitale wratten en vulvaire hooggradige squameuze intra-epitheliale lesies (vulvaire HSIL).

Huidwratten zijn goedaardig en worden in de meeste gevallen veroorzaakt door HPV type 1, 2, 27 en 57. Ze verdwijnen meestal spontaan binnen 2 jaar na diagnose, maar kunnen veel fysieke en psychosociale klachten geven. Genitale wratten, voornamelijk veroorzaakt door HPV type 6, zijn zeer besmettelijk en is wereldwijd een van de meest voorkomende seksueel overdraagbare aandoening (SOA). Genitale wratten kunnen klachten geven zoals pijn en jeuk, maar het voornaamste probleem is de psychosociale belasting van aangedane mensen. Vulvaire HSIL is een voorstadium van schaamlipkanker en wordt veroorzaakt door hoog-risico HPV types, in de meeste gevallen HPV type 16. Veel voorkomende symptomen zijn jeuk en pijn, seksuele problemen en psychosociale klachten.

Bovengenoemde HPV-geïnduceerde aandoeningen komen steeds vaker voor. Omdat de huidige behandelingen niet altijd werken en vervelende bijwerkingen kunnen hebben, en vanwege de hoge kans op het terugkomen van de aandoeningen, is onderzoek naar nieuwe behandelingen noodzakelijk.

De klassieke ontwikkeling van nieuwe medicijnen kent verschillende fases van onderzoek, waarbij in de vroegere fase de veiligheid wordt onderzocht en pas in een latere fase de effectiviteit van het middel in patiënten. Dit heeft als gevolg dat een nieuw middel bij voorbaat een lage slagingskans heeft in de geneesmiddelen ontwikkeling omdat pas in een latere fase blijkt of het middel effectief is voor een bepaalde ziekte. In dit proefschrift hebben we onderzocht of een vraag gerelateerde aanpak van geneesmiddelontwikkeling een efficiëntere aanpak zou kunnen zijn voor door HPV-geïnduceerde ziekten. Tijdens de studies hebben we de volgende vragen gesteld:

1 Komt het middel aan op de plaats van werking, ofwel wordt het op de lesies aangebracht?
2 Heeft het middel de beoogde farmacologische werking?
3 Heeft het middel gunstige effecten op de ziekte?

Met deze aanpak konden wij nieuwe instrumenten ontwikkelen en invoeren in de klinische studies die beschreven staan in dit proefschrift. In de toekomst kunnen deze bijdragen aan het verbeteren van de ontwikkeling van medicijnen en hopelijk aan meer effectieve behandelingen van HPV-geïnduceerde aandoeningen.

HET ELEKTRONISCHE DAGBOEK

De eerste vraag van de vraag gerelateerde aanpak was: komt het middel aan op de plaats van werking? De meeste geneesmiddelen voor HPV-geïnduceerde ziekten worden direct op de lesies aangebracht. Het is bekend dat de therapietrouw (de mate waarin patiënten zich houden aan de voorschriften voor het gebruik van geneesmiddelen) bij deze middelen erg laag is. We hebben een elektronisch dagboek (e-dagboek) ontwikkeld om te onderzoeken of patiënten het middel aanbrengen op de lesies (**hoofdstuk 1**). Het e-dagboek liet een hoge therapietrouw zien, mogelijk door een ingebouwde herinnering in het e-dagboek die de patiënten motiveerde om het middel op de afgesproken tijd aan te brengen. Het e-dagboek werd door de patiënten als zeer gebruiksvriendelijk ervaren. Ook konden patiënten met behulp van het e-dagboek symptomen van de ziekte rapporteren. We kunnen concluderen dat het e-dagboek een goed hulpmiddel is om te meten of het medicijn de plaats van werking bereikt, en kan leiden tot een betere therapietrouw.

VIRALE LADING

De tweede vraag van de vraag gerelateerde aanpak was: heeft het middel de beoogde farmacologische werking? Het testen van de farmacologische werking van het middel helpt ons bij het voorspellen van de werkzaamheid. Hiervoor is de hoeveelheid van het virus in de lesies gemeten, ook wel virale lading genoemd. Dit zegt iets over hoe actief het virus aan het delen is. De bepaling en monitoring van deze virale lading is van grote waarde voor de voorspelling van de werkzaamheid van een geneesmiddel. De gouden standaard voor het bepalen van de virale lading is een biopt, maar dit is een invasieve handeling die de plek (gedeeltelijk) wegneemt en slechts één of een beperkt aantal keren kan worden uitgevoerd. Een alternatieve methode is een huid uitstrijkje van de lesies. Eerder werd al aangetoond dat het huid uitstrijkje betrouwbaar kan laten zien welk HPV type er aanwezig is. Tot op heden is nog niet aangetoond hoe betrouwbaar de virale lading kan worden gemeten met een huid uitstrijkje, in vergelijking met een biopt. We hebben het huid uitstrijkje in 3 verschillende klinische studies uitgevoerd (**hoofdstuk 3, 4 en 5**) en gevonden dat, ondanks dat de virale lading erg verschilde per patiënt, het huid uitstrijkje een goede weergave gaf van de virale lading voor en na verschillende behandelingen over de tijd. Uit de studie met huidwratten bleek dat de virale lading gebaseerd op een biopt en een huid uitstrijkje vergelijkbaar was. Het meten van de virale lading met een huid uitstrijkje lijkt dus een geschikte manier om de werkzaamheid van een middel te bepalen.

DRIEDIMENSIONALE (3D) FOTOGRAFIE

De derde en laatste vraag die we wilden beantwoorden, was: heeft het middel gunstige effecten op de ziekte? Om de effectiviteit van een middel op HPV-geïnduceerde lesies te kunnen bepalen, is het belangrijk om de lesies nauwkeurig te meten over de tijd. Vooral bij vulvaire HSIL is het soms moeilijk om de precieze afmetingen van lesies te bepalen. Een instrument welke lesies beter kan visualiseren kan daarom van grote waarde zijn voor het vervolgen van de lesies tijdens de behandeling, maar ook voor de vroege detectie van lesies. Driedimensionale (3D) fotografie kan mogelijk nauwkeurig inzicht geven in de afmetingen van lesies. 3D fotografie wordt al in verschillende vakgebieden gebruikt, zoals in de plastische chirurgie, maar wordt nog niet toegepast voor de ontwikkeling van nieuwe geneesmiddelen voor HPV-geïnduceerde lesies. Met 3D fotografie zou de effectiviteit mogelijk al vroeg kunnen worden voorspeld op basis van kleine veranderingen in de vorm of afmetingen van lesies. In **hoofdstuk 3** is het gebruik van 3D fotografie onderzocht voor HPV-geïnduceerde lesies in de klinische studies beschreven in dit proefschrift. 3D fotografie liet een hoge nauwkeurigheid zien, ook als de foto's werden beoordeeld door verschillende personen. De gouden standaard voor het opmeten van lesies is een schuifmaat. De vergelijking van de schuifmaat met 3D fotografie liet kleine verschillen zien voor het bepalen van de diameter van huidwratten, genitale wratten en vulvaire HSIL en de hoogte van huidwratten. 3D fotografie blijkt ook toepasbaar voor het opmeten van de oppervlakte en het volume van lesies. Op grond van de beschreven studies kunnen we concluderen dat we met 3D fotografie de werkzaamheid van een middel mogelijk al in een vroege klinische fase kunnen voorspellen.

NIEUWE BEHANDELINGEN VOOR HPV-GEÏNDUCEERDE ZIEKTEN

In dit proefschrift werden twee nieuwe geneesmiddelen voor de verschillende HPV-geïnduceerde ziekten onderzocht: 1) omiganan en 2) ionische contra virale therapie (ICVT).

Omiganan is een antimicrobieel peptide met immuunmodulerende en antivirale eigenschappen en werd onderzocht bij patiënten met genitale wratten en vulvaire HSIL (**hoofdstuk 4**). Omiganan bleek veilig te zijn voor beide indicaties en verlaagde de virale lading bij patiënten met genitale wratten, maar niet bij patiënten met vulvaire HSIL. Dit middel zorgde bij beide indicaties echter niet voor een afname van de lesies.

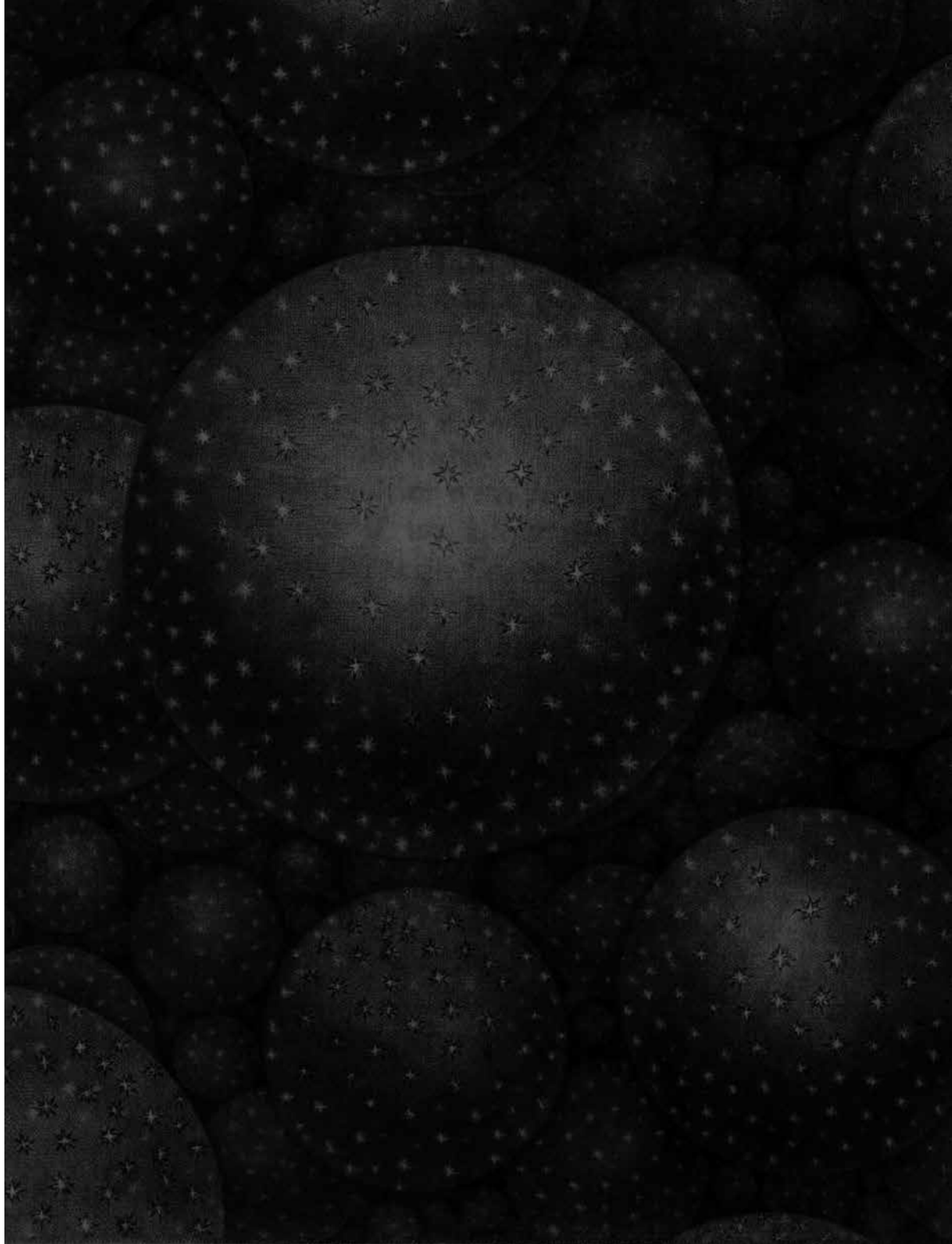
ICVT bestaat uit digoxine en furosemide en remt de kaliuminstroom die DNA-virussen nodig hebben om te kunnen delen. We onderzochten de veiligheid en effectiviteit van ICVT bij patiënten met huidwratten (**hoofdstuk 5**) en vonden dat ICVT veilig gebruikt kon worden voor deze indicatie en leidde tot een verlaagde virale lading en een afname van de grootte van de huidwratten. Een vervolgstudie met ICVT in patiënten met genitale wratten (**hoofdstuk 6**) liet echter geen farmacologische activiteit en ook geen klinische effectiviteit zien van ICVT.

CONCLUSIE EN TOEKOMSTPERSPECTIEVEN

De beschreven instrumenten in dit proefschrift kunnen van grote waarde zijn voor een efficiëntere ontwikkeling van geneesmiddelen voor de behandeling van HPV-geïnduceerde ziekten. De vier beschreven klinische onderzoeken

met de nieuwe middelen omiganan en ICVT bij patiënten met verschillende HPV-geïnduceerde ziekten toonden aan dat beide middelen een verschillende farmacologische en klinische werkzaamheid hebben, afhankelijk van de HPV-geïnduceerde ziekte.

In toekomstige klinische onderzoeken zullen de patiënt gerapporteerde symptomen in het e-dagboek verder onderzocht moeten worden en ook zal het e-dagboek van waarde kunnen zijn in de dagelijkse praktijk om de behandeling te optimaliseren. Voor de vergelijking van huid uitstrijkjes en biopten voor de bepaling van virale lading bij vulvaire HSIL en AGW zal nog meer onderzoek gedaan moeten worden om aan te tonen dat de biopten vervangen kunnen worden door huid uitstrijkjes. En voor implementatie van de 3D fotografie is validatie van oppervlakte en volume in HPV-geïnduceerde lesies waardevol. Het bepalen van de afmetingen van vulvaire HSIL was zowel met de schuifmaat als de 3D fotografie soms lastig, voornamelijk door het variabele uiterlijk van de lesies en de vaak onscherpe begrenzing. Het zou voor toekomstige klinische onderzoeken ideaal zijn om de visualisatie van deze lesies te verbeteren, bijvoorbeeld met een specifiek fluorescerend ligand. Het beter visualiseren van deze lesies zou zowel voordeel hebben tijdens klinische onderzoeken als in de dagelijkse praktijk voor de diagnostiek en tijdens operatieve ingrepen.



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Curriculum vitae

Melanie Rijsbergen was born on the 13th of December 1987 in Leiderdorp, the Netherlands. She graduated from secondary school in 2006 at the Da Vinci College in Leiden. In the same year she started with Biomedical Sciences and in 2006 she started to combine this study with Medical school, both at the Leiden University. She performed her graduation project at the department of Gynecology and Obstetrics at the Leiden University Medical Center (LUMC) where she studied the STAT phosphorylation upon cytokine stimulation and co-inhibitory molecule expression in vulvar HSIL patients. In April 2014 she graduated as Master of Biomedical Sciences and Medical Doctor and started her professional carrier as a physician at the department of Gynecology and Obstetrics in the HagaZiekenhuis, The Hague. In July 2015 she started working as PhD student and research physician at the Centre for Human Drug Research (CHDR) under supervision of Robert Rissmann, Mariëtte van Poelgeest and prof. J. Burggraaf. As part of her research training, she was trained as a clinical pharmacologist and obtained her degree in clinical pharmacology in 2019. In January 2020 she started her residency gynecology in the Onze Lieve Vrouwe Gasthuis (OLVG) and the Amsterdam Medical Center (AMC). In 2010 she met her fiancé Michel Crama with whom she lives together in Oegstgeest with their two children Tim (2016) and Suus (2018).

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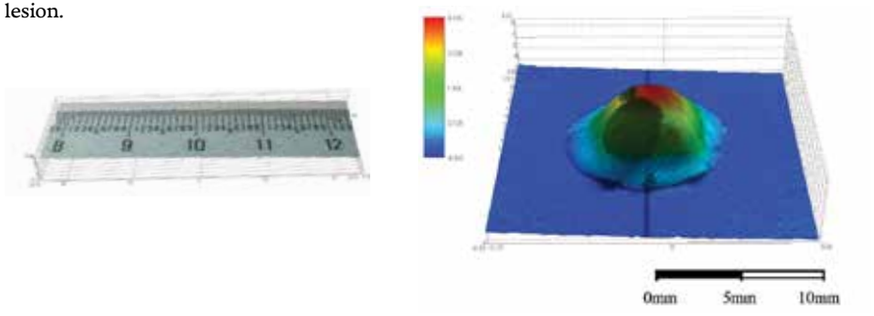
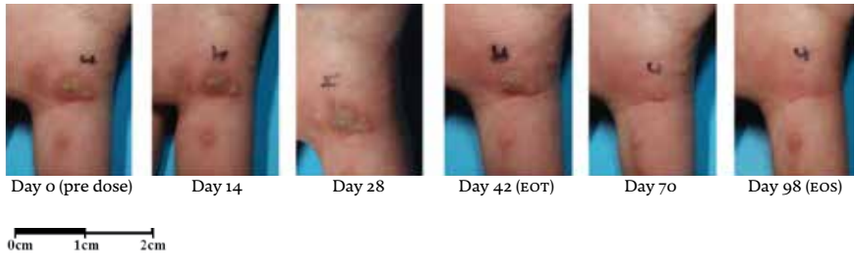
SEE INSIDE FOR COLOR ILLUSTRATIONS OF CHAPTER 3, 4 AND 5

Chapter 3 – Figure 2. Three-dimensional reconstruction by stereophotogrammetry. A representative lesion for all three HPV-induced lesions (cutaneous warts, anogenital warts and vulvar HSIL) with on the left the 2D photograph, in the middle the 3D reconstruction and on the right the heat map showing height differences and the manual contour around the lesion.



Chapter 4 – Figure 2. Photography assessments of lesions over time. Photography of a patient with angenital warts and vulvar HSIL, both patients were treated with omiganan. Pre-dose (day 0) the lesions are clearly visible. Upon treatment, the genital warts clearly resolve (A) and the vulvar HSIL remained the same (C). The patient of picture 2a had total clearance of the genital warts at EOS, but a post-inflammatory hypopigmentation has occurred at the lesion site. Day 0 is before start of treatment, day 84 is at the end of treatment (EOT) and day 168 is at the end of study (EOS).

Chapter 5 – Figure 3c. Photography assessments of a common wart of subject #6 (digoxin+furosemide). (A) Analysis of the primary endpoint for the intention-to-treat population (N=79) was performed using a mixed model with treatment, time and treatment by time as fixed factors and subject as random factor. All statistical tests were two-tailed with α-level of 0.05. Results showed a statistically significant reduction of wart size in the digoxin+furosemide group compared to placebo (-2.5mm; 95% CI -4.9 to -0.1; p=0.04). Single treatment groups (digoxin vs placebo and furosemide versus placebo) showed no statistically significant effects (-1.5mm; 95% CI -3.9 to 0.9; p=0.21, and -1.1mm; 95% CI -3.4 to 1.3; p=0.38, respectively). Changes in diameter were most pronounced after EOT, as shown in Figure 3A. (B) In the analysis of all treated warts (N=139) a statistically significant wart size reduction was observed between each active treatment group and placebo (digoxin+furosemide versus placebo; -3.0mm; 95% CI -4.9 to -1.1; p=0.002, digoxin vs placebo -1.9mm; 95% CI -3.7 to -0.2; p=0.03, furosemide versus placebo -2.1mm; 95% CI -3.8 to -0.4; p=0.01). (C) A photography assessment of a treated wart in the digoxin+furosemide group.



Chapter 5 – Figure 5. Histological representative cases of classical cutaneous viral warts. (A) Verruca vulgaris H&E low power view (50x) with architectural characteristic inturning of the elongated rete ridges, epidermal hyperplasia, papillomatosis, hypergranulosis, hyperkeratosis and columns of parakeratosis. (B) Verruca vulgaris H&E, detail view (200x): note koilocytes (arrowhead) and coarse granula (arrows) mostly in top layers (stratum granulosum). (C) H&E low power view (50x) of verruca plana with epidermal hyperplasia, hypergranulosis, hyperkeratosis, koilocytes in middle and upper layers. (D) verruca plana H&E, detail view (100x) note the absence of papillomatosis, parakeratosis and coarse granula.